IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):	Victor Raso
Application No.:	09/992,994
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Title:	IMMUNOLOGICAL CONTROL OF β-AMYLOID LEVELS *IN VIVO*
Art Unit:	1652
Examiner:	Patterson, C.

DECLARATION OF VICTOR A. RASO, Ph.D.

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Vic Raso, do hereby declare and say:

I. From before December 2, 1997 until June 16, 1999, I was attempting to obtain funding to support myself, my family, and my research

1. I am a Senior Scientist, without tenure, at the Boston Biomedical Research Institute (BBRI). I joined the BBRI in 1988 as a Visiting Scientist with a one-year term. [Ex. A]. I became a Senior Scientist in 1989 with a five-year term [Ex. B] and was re-reappointed as a Senior Scientist in 1994, 1999, and 2004 for additional five-year terms. [Ex. C].

2. The BBRI is an independent, not-for-profit biomedical research institute. As such, it is dependent upon federal grants, principally from the National Institutes of Health (NIH), for its revenues. As a faculty member, I was expected to be self-funding,

generating grants to fund my laboratory, provide indirect costs for running the institute including my own salary and fringe benefits. Each of my offer letters indicated that my salary was contingent on his ability to generate outside funding. None of my appointments were tenured. [Ex. A, Ex. B and Ex. C].

3. Because I do not have tenure, I must be reappointed at the end of each term. Reappointment to an additional term is dependent upon 4 criteria. First and foremost among these criteria is grant support. [Ex. D].

4. The BBRI provides temporary Bridge Support to faculty members who lose funding but are thought to be likely to regain funding. [Ex. E at p. A-22]. Bridge Support is available for only one year, other than in "exceptional circumstances." [Ex. D at p. E-06]. The primary criteria for determining whether such an exceptional circumstance existed is the probability of funding. [Ex. D at p. E-06].

5. I ran out of funding in 1995 and started to receive Bridge Support. BBRI changed its policy on Bridge Support in 1996. Still having no outside funding, I requested a second year of Bridge support in late 1996. As my request indicated, I was actively working on five different grant applications. [Ex. F at p. 1]. Although I may not have met the new criteria for bridge support, BBRI approved my further bridge support, although only for 8 months, not a full year. [Ex. G at p. 2].

6. Despite my continuing attempts to obtain funding, I did not obtain any. I even tried to obtain other sources of funding, seeking Small Business Innovation Research Grants, as well as grants from the Alzheimer's Association. Having failed to get the typical NIH grants, I was becoming quite concerned. I knew the BBRI was not terribly interested in such grants because such grants do not provide enough indirect costs.

Indeed, I was a member of the Committee on Research (COR) that voted to grant further bridge support to a faculty member. [Ex. G at p. 1]. That vote was contingent on the faculty member obtaining NIH funding. The COR specifically stated that "an Alzheimer's [Association] grant would not be enough." [Ex. G at p. 1].

7. Nonetheless, I applied for these grants as well. I needed some funds to conduct my research, and I thought that securing other sources of funding would be helpful in obtaining a subsequent NIH grant. Besides not having any current grants, I had not worked in the Alzheimer's field before. The reviewers of my grant applications indicated that this was one reason they were rejecting my grant applications.

8. My Bridge Support was not sufficient to keep my laboratory running. I maintained my technician in 1997 only by cutting my salary to pay for her salary. [Ex. H at p. 1]. I thought it was particularly important to keep her to obtain the data for the grants I was writing. Indeed, I could not do much of the needed lab work because I was "increasingly pulled away from the laboratory to write grants and papers." [Ex. H at p. 2].

9. I continued to fail to obtain funding. Therefore, in September 1997, the Director of the BBRI formally warned me that I might not be reappointed as a Senior Scientist when my term ran out in two years. [Ex. I]. The Director indicated that my continued failure to obtain NIH grants might cause the BBRI to not renew my appointment. She also told me that she hoped I could obtain "NIH or similar grants," which might allow me to be reappointed in two years. [Ex. I].

10. It became absolutely clear to me that I needed to obtain NIH funding to have any chance of retaining a position at the BBRI.

11. Also, BBRI allocates laboratory space based upon funding and utilization levels. [Ex. D at p. B-06a]. Besides warning me that I might not be reappointed, the Director also reminded me of this policy. [Ex. I]. Separate from my academic appointment as a Senior Scientist, if I did not have funding, I was at risk of losing some or all of my laboratory space.

12. It became absolutely clear to me that I needed to obtain NIH funding to have any chance of retaining adequate laboratory space. Indeed, I eventually lost all my space except for one-half a laboratory bench and my office. My lost laboratory space was remodeled for a new faculty member.

13. The BBRI requires monthly Time and Effort Reports of all faculty members. [Ex. D at p. A-06]. The reports show the percentage of time spent on each fund. Funds normally correspond to particular grants received by the faculty member. My Bridge Support—and salary—ended on September 15, 1997. At that time, my time and effort reports, switched from Institutional Support to Grant Writing. As the Time and Effort Reports indicate, I spent all my time preparing grants from the end of my Bridge Support on September 15, 1997, through December 31, 1997.

14. I was terminated at the end of 1997 because I did not have any funds to support my work. [Ex. J and Ex. K]. Upon termination, I was no longer eligible for health insurance through BBRI. As a faculty member, however, I was still entitled to use BBRI facilities until such time as they allocated my laboratory space to another, funded faculty member.

15. My grant writing finally began to pay off in April 1998, when I received a small SBIR grant from the NIH. Such a grant is of short duration and did not sufficiently cover

BBRI's overhead. Accordingly, the Director met with me and informed me that she would be bringing faculty candidates by to look at my laboratory space, indicating that it would be available for them. [Ex. L]. She indicated the BBRI might be able to accommodate me in the basement.

16. The BBRI had some laboratory space in the basement, which was generally the least desired. Faculty members who were transitioning out of the BBRI were sometimes placed there temporarily. Indeed, the faculty member mentioned above whom the COR voted contingent bridge support was moved to that space before leaving the BBRI.

II. My grant-writing efforts during this time

17. During this time, I was constantly working on grant applications. From January 1997 until August 1999, I prepared and submitted 16 grant applications. These 16 grant applications all concerned Alzheimer's Disease, particularly the use of antibodies to β-amyloid:

Date	Type of Grant (Form)	Title	Bates Number
01/15/1997	NIH (PHS 398)	AMYLOID β PEPTIDE EQUILIBRIA IN "ALZHEIMER'S MICE"	B000030 [Ex. P]
08/29/1997	NIH; SBIR (6246-1)	IMMUNOTHERAPY OF ALZHEIMER'S DISEASE	B000093 [Ex. Q]
10/21/1997	NIH (PHS 398)	AMYLOID β PEPTIDE EQUILIBRIA IN "ALZHEIMER'S MICE"	B000223 [Ex. R]
12/11/1997	NIH; SBIR (PHS 6246-1)	CEREBRAL ANTIBODY DELIVERY TO TREAT ALZHEIMER'S DISEASE	B00269A-269Y [Ex. S]
01/26/1998	NIH (PHS 398)	CEREBRAL DELIVERY OF VECTORIZED ANTI-β-AMYLOID ANTIBODY	B000270-312 [Ex. T]

04/13/1998	NIH; SBIR (PHS 6246-1)	CATALYTIC ANTIBODIES TO INACTIVATE β-AMYLOID	B000313-339 [Ex. U]
05/20/1998	NIH (PHS 398)	VACCINE TO MODULATE SYSTEMIC β-AMYLOID LEVELS	B000340-365 [Ex. V]
05/27/1998	NIH (PHS 398)	PROBE TO VISUALIZE CEREBRAL β-AMYLOID PLAQUES	B000366-392 [Ex. W]
09/01/1998	Alzheimer's Association (KRS)	VACCINE TO MODULATE (SYSTEMIC) β-AMYLOID LEVELS	B000432-449 [Ex. X]
09/03/1998	Alzheimer's Association (KRS)	CEREBRAL DELIVERY OF VECTORIZED ANTI-β-AMYLOID ANTIBODY	B000393-413 [Ex. Y]
09/04/1998	Alzheimer's Association (KRS)	CATALYTIC ANTIBODIES TO INACTIVATE β-AMYLOID	B000414-431 [Ex. Z]
10/11/1998	NIH (PHS 398)	CEREBRAL DELIVERY OF VECTORIZED ANTI-B-AMYLOID ANTIBODY	B000476-523 [Ex. AA]
01/19/1999	NIH (PHS 398)	IMMUNOTHERAPEUTIC AGENTS TO TREAT ALZHEIMER'S DISEASE	B000141 [Ex. AB]
01/27/1999	NIH (PHS 398)	NOVEL TRANSITION STATE PEPTIDE ANALOG ANTIGENS	B000524-569 [Ex. AC]
04/12/1999	NIH; SBIR (PHS 6246-1)	BIPHASIC PROBE TO VISUALIZE INTRACELLULAR B-AMYLOID	B000450 [Ex. AD]
08/13/1999	NIH; SBIR II (PHS 6246-2)	IMMUNOTHERAPY OF ALZHEIMER'S DISEASE	B000187 [Ex. AE]

18. I believe this is an exceptional number of grant applications to prepare and submit in about 2 ½ years. The NIH's guidelines indicate that each grant application should take three to six months to write. [Ex. M]. Despite my lack of salary from BBRI, decreasing laboratory space, and eventual lack of a technician, I prepared and submitted so many grant application because I was desperately trying to save my career at BBRI so that I could conduct my research. I worked many nights and weekends writing the grant applications and obtaining the data to be included in them.

III. My experimental work during this time was devoted to an Alzheimer's vaccine

19. Grant applications require data. Accordingly, I conducted many experiments to support these grant applications. Because of my circumstances at BBRI, however, the focus of those experiments was simply to obtain data needed for the grant applications. Without funding, I did not have time to perform extensive experiments nor keep detailed notes. I was simply trying to obtain the data necessary to prepare and submit grant applications, which I hoped would be granted to provide for my family and to save my career.

20. The experiments conducted in support of the grant applications are summarized in the attached chart. [Ex. N]. The laboratory notebook pages and printouts containing the data from these experiments are also attached. [Ex. O]. I believe these documents are readily understood by a scientist in the field. However, I provide the following explanation of the experiments I performed for further clarification. Unlike other principal investigators, I had to perform most, if not all, of these tasks myself because of my lack of funding and space at the BBRI.

21. My experiments encompassed active as well as passive immunization for the treatment and/or prevention of Alzheimer's disease. The active vaccines I developed included both naturally occurring β-amyloid peptides as vaccines to elicit conventional (binding) antibodies and transition state analog β-amyloid peptide vaccines to elicit catalytic antibodies that bind and hydrolytically cleave β-amyloid into harmless fragments. For passive immunization I initially injected either conventional binding antibodies directed against naturally occurring β-amyloid peptides or catalytic antibodies directed against transition state analog β-amyloid peptides.

22. Work on all four of these basic concepts was simultaneously initiated before December 2, 1997 when I synthesized the first multiplex peptide cocktail. I designed that preparation to contain both natural and transition state analog β-amyloid peptides. I proceeded to use that formulation as an active vaccine in normal and transgenic mice so that both conventional and catalytic antibodies would be induced. I subsequently derived monoclonal antibodies from immunized mice and utilized those anti-β-amyloid antibodies as passive vaccines.

A. Materials and Methods

1. Monoclonal Antibody Production

23. A key aspect of my research involved monoclonal antibodies. Consequently, a significant proportion of time and effort was devoted to formulating strategies for new antibodies and antigens, generating numerous lines of monoclonal antibodies, assaying for their specific activities, isolating the lines of interest, and measuring the quality and quantity of each individual line.

24. The laboratory procedure for generating monoclonal antibodies is well established in the field. However, obtaining a high quality monoclonal antibody of interest was a time-consuming process each time. A mouse was immunized by injection of an antigen X to stimulate the production of antibodies targeted against X. The antibody forming cells were then isolated from the mouse's spleen. Monoclonal antibodies were produced by fusing single antibody-forming cells to tumor cells grown in culture. The resulting cell is called a hybridoma. Each hybridoma produces relatively large quantities of identical antibody molecules. By allowing the hybridoma to multiply in

culture, it was possible to produce a population of cells, each of which produces identical antibody molecules. These antibodies are called "monoclonal antibodies" because they are produced by the identical offspring of a single, cloned antibody producing cell. The production of hybridoma generally involved the following steps:

1. Immunization of mice (in vitro or in vivo)
2. Spleen removal and preparation of a single cell suspension
3. Myeloma cell preparation
4. Fusion of spleen cells and myeloma cells
5. Post-fusion cells cultured in hybridoma selection medium (HAT)
6. Collection and dispersion of peritoneal macrophages and/or the use of conditioned medium.
7. Addition of fused cells to microtiter plates with macrophages 48 hours post-fusion (2-4x 10^6 cells/ml)
8. Culture of cells: 37^0C, 5% CO2 [feed with HAT medium]
9. 7 to-21 days post fusion: observe and enumerate hybridoma clones
10. Screen for specific antibody production
11. Expand cultures positive by screening test
12. Reclone by a limiting dilution technique all positive hybridoma clones to assure monoclonality and to select for the fastest growing cell line with the greatest antibody production. Hybridomas would be recloned periodically (after 3-4 months of culture) to prevent overgrowth of the preferred culture by mutants or cells expressing an altered phenotype.

13. Inject 2-10x10^6 recloned hybridoma cells into BALB/c mice which had received an i.p injection of 0.3 ml of pristane 7 days previously. Collect ascites 7-21 days latter.

14. Guard against loss of the hybridoma cell line by storing several amps of each clone in liquid nitrogen.

Related records from Christine Kearney's lab notebooks are attached. [Ex. O]. Many of these records were made in 1996, when the basic laboratory protocols were established, and thereafter followed.

25. Because these time-consuming steps were routinely carried out, no specific entries were recorded in a laboratory notebook on a daily basis. Some records are available which noted the final phase of screening of hybridoma lines that presented "promising" characteristics, and experimental data showing the results of the ELISA assays identifying these lines, and eventually leading to the successful isolation of the monoclonal antibodies that exhibited potentially therapeutic activities for Alzheimer's disease, as claimed in the invention of the patent application at issue. Therefore, it should be inherently appreciated that considerable amount of time had been spent on the earlier phase of the antibody production, despite the fact that few entries were recorded (as being routine procedures).

26. The attached documents document the production of numerous monoclonal antibodies [Ex. O], as identified in the attached chart. [Ex. N]. The above-step necessarily occurred each time. Otherwise, the monoclonal antibody could not have been created.

2. Cell Culture

27. Besides the hybridomas used to create monoclonal antibodies, several lines of cells grown in culture were used in the experiments I conducted. Maintaining cell cultures involves some tasks on a daily basis, such as changing culture medium for each dish of cells, preparing and maintaining growth media and other solutions and reagents needed for culturing cells, and monitoring the growth state of the cells. In addition, for the propagation of cell lines, other components of maintenance are involved.

28. I used several different types of cultured cells for different purposes. The time required to maintain cells in culture was between less than an hour up to several hours per day. Most cell lines grown in culture require maintenance and routine care approximately every 2-3 days. When an experiment needed to be started from a frozen stock of cells, a vial of cryo-preserved cells were thawed and placed in an appropriate growth medium. Cells were then allowed to proliferate until they reach a desired number of cells/ml medium or confluence (density or fullness per given growth surface, e.g., a culture dish). Depending on the growth rate of the particular cell type, this could take up to one week or sometimes longer before I was able to use the plate of cells for an experiment.

29. The attached documents document the production and/or maintenance of numerous cell-line cultures [Ex. O], as identified in the attached chart. [Ex. N]. [B001559-1561] The above-steps necessarily occurred each time. Otherwise, the cell cultures could not have been created or maintained.

3. **Animal Protocols**

30. I was trained in protein biochemistry and was familiar with many of the classic biochemical techniques and protocols, as well as the laboratory equipment used to perform these procedures. During the course of my work on the present invention, I had to learn a number of new methods and techniques. Among them, I needed to familiarize myself with general animal handling procedures, namely, the breeding of mice and the maintenance of the mouse colonies. In particular, the initial phase of establishing a colony of mice was challenging. New animals were generally placed in quarantine for several weeks in order to minimize potential cross-infection. Upon being deemed free of contaminants, the animals were then allowed into the main facility. Before any experiments could be performed using the mice, I had to establish a new colony from the initial set of mice.

31. Each of my animal experiments required many subjects in order to compensate for individual variations and errors. To accommodate that requirement, we had to expand the colony to sufficient size and that took lots of time. Because the gestation period for mice is approximately 18-20 days, it would take, even under ideal circumstances, almost three weeks to obtain the first set of litters. Upon arrival of a litter, each mouse would be tagged, then "genotyped" (i.e., genetically determined by DNA analysis) to confirm its genetic profile. The results of this process would simply be recorded notebook as "positive (+)" or "negative (-)" dependent upon whether it had acquired the human β-amyloid gene associated with AD.

32. My first attempts to expand a mouse colony stumbled initially, likely due to my inexperience with the procedures. Nevertheless, eventually I managed to obtain a

colony of the mice. Generally, mice reach maturity at the age of approximately 5-7 weeks. At this point, they could be used for certain experiments; alternatively, they could be used for further breeding.

33. Another challenge I discovered in animal experimentation is to obtain enough mice that are "age-matched" "background-matched" and "sex-matched", in order to obtain consistency in data acquisition. In particular, some of my experiments using animal models involved "aged" (~1+ year old) mice in which the age-dependent manifestation of Alzheimer-like disease was mimicked. To perform these experiments, it naturally required one year or longer after the arrival of a litter. For these reasons, it often takes a relatively large colony of mice to sustain a much smaller number of animals actually being used in experiments. This was particularly difficult as the adult mice tended to eat their newborn pups.

34. In addition, my experiments involved a series of immunizations (injections) of mice. Typically, a first injection of antigen to trigger immune response ("immunization") was given to an animal; subsequently, about four to six weeks later a second immunization (called "boost") is given. The right interval between these injections is important in ensuring effective immune response in the mouse. Therefore, it would take a minimum of approximately two months from the time of the initial injection before I could obtain an animal that exhibited a robust immune response to a given β-amyloid antigen.

35. The attached documents record the production and/or maintenance of the mouse colony [Ex. O], as identified in the attached chart. [Ex. N]. [B001760-1795] The

above-steps necessarily occurred each time. Otherwise, the colony could not have been created or maintained.

36. Similarly, the attached documents record the immunization of mice [Ex. O], as identified in the attached chart. [Ex. N]. The above-steps necessarily occurred each time. Otherwise, the immune responses could not have been generated and boosted.

4. Histology

37. Histological analyses of tissues and organs involved a multitude of procedures. This was also an area of discipline that involved new techniques that I had to acquire.

38. In general, histology involved the following steps, with some modifications: (1) Dissection/removal of tissue specimen of interest, (2) fixation of the tissue to preserve the integrity of the specimen, (3) post-fixation procedure such as dehydration with sucrose solutions, (4) freezing, (5) cryostat sectioning (6) mounting and slide preparation, (7) staining and other labeling procedures, (8) microscopy (light microscopy, confocal microscopy, fluorescent microscopy, etc) for label detection and morphological analysis. In order to learn these techniques, I used normal/wild type mice for 'practice' and continued until I was able to consistently perform the histological procedures with a reasonable degree of reliability and reproducibility.

39. The attached documents document the histological analysis of mice [Ex. O], as identified in the attached chart. [Ex. N]. The above-steps necessarily occurred each time. Some of the preliminary data were also included in my grant application submitted in October of 1998 (B000490). Otherwise, the data could not have been obtained.

5. Capillary Depletion Experiments and Other In Vivo Procedures

40. I used a technique commonly referred to as "capillary depletion" to demonstrate *in vivo* transcytosis of vectorized antibodies across the blood brain barrier of the mouse. In short, this experiment addressed the question of whether a compound of interest crosses the blood brain barrier into the brain parenchyma of an animal by separating the vascular endothelium from the brain parenchyma by centrifugation and measuring radiolabel associated with each of the pools. The experiment is technically extremely challenging. In addition, the procedure required the use of radio-labeled compound to be introduced into an animal, and subsequent handling of the radioactive tissue samples for the centrifugation step as well as for isolation of separated layers, followed by scintillation counting.

41. The experimental setup for terminal surgery (i.e., the animal is killed for tissue harvesting and histological analyses) would take about one hour. The experimental procedures would be accomplished within one hour per mouse. The brain preparation and tissue harvesting would take about one-half hour per mouse. If radiolabeled test compounds were used, the cleanup time could take as much as an hour to comply with appropriate radiation safety protocols.

42. The experimental setup for the pharmacokinetic study would then take about one hour. The study itself would be accomplished within about one hour to 48 hours per mouse, depending on the pharmacokinetic sampling protocol. Tissue harvesting and cleanup would require another hour per mouse, depending on whether radiolabeled substrates are used.

6. Graphics and Image Processing Workstation

43. To visualize and analyze several of the experimental results, I learned to use the BBRI's graphics and image processing workstation. The workstation is a computer system that integrates application programs that allow the user to model and manipulate various mathematical, geometrical, and structural information into two- and three-dimensional graphic information. For biomedical and biological research purposes, the graphic workstation can be used to, for example, enter/input image data, extract aspects of information based on one or more variables and parameters of choice, and analyzing the information. I would input a scanned image of a cross-section of a mouse specimen histologically stained or radioactively labeled. The degree of intensity of the signal was indicative of the relative amount of for example, β-amyloid or anti-β-amyloid antibodies in the sample. I then quantitatively analyzed and graphically manipulated (e.g., using gradients of pseudo-colors, etc.) the data.

44. In addition, I used the workstation to model chemical structures of β-amyloid peptides at the atomic and or molecular levels. Using these features, I could graphically represent a molecular or chemical structure of a known or newly synthesized β-amyloid peptide. The workstation also allowed energy calculations and structural/conformational analyses of peptides. I used the graphics workstation to perform energy and conformational analyses of transition state verses native Alzheimer's disease-associated peptides.

45. Using of the workstation and associated software programs was quite complicated. I had a demonstration of basic features of the system by colleagues,

followed by an extensive trial-and-error process to obtain results. I learned how to use the graphic workstation for the purpose of modeling, analyzing and representing molecular structures for my research over the course of many months.

46. Unlike specific experiments being conducted, it is more difficult to point out the exact date and time when I was learning to use the workstation to further my research and grant-writing, although it was quite significant. I spent many hours, quite possibly as many as one hundred hours, using and learning the graphic workstation, and many of these sessions may not have yielded actual data or analyses of data to which the exact time of use can be traced.

47. The attached documents record the graphics workstation analyses [Ex. O], as identified in the attached chart. [Ex. N]. The above-steps necessarily occurred before this work. Otherwise, the data could not have been obtained.

B. Specific β-amyloid Alzheimer's Vaccine Experiments

48. Before December 2, 1997, I had conceived of β-amyloid active and passive Alzheimer's vaccines, and I had generated a number of hybridoma cell lines in an effort to establish useful monoclonal antibodies to β-amyloid ("Alz PS"). I had obtained ELISA assay results, showing some positive and negative results for the Alz PS clones. (B001450). I continued to screen for monoclonal antibodies by ELISA assays. (B001451).

49. Before December 2, 1997, I had successfully used those monoclonal antibodies in an antibody:polyethylene glycol (PEG) assay that measured the binding of

native, human full-length radioactive (^{125}I) β-amyloid to monoclonal antibodies 5A11, 2E3, 5G11, 2H11, 11H2. (B001452-B001453).

50. Human β-amyloid exists in several forms and, as a native protein, may not be expected to be antigenic. Thus, before December 2, 1997, I synthesized a mixture of four peptides encompassing amino acids 10-25 of native human β-amyloid plus a cysteine for thioether coupling to an antigenic carrier. These peptides included the native human sequence (Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly) plus three modified versions in which the two phenylalanine residues were randomly substituted with a statine transition state analog of phenylalanine ((3S, 4S)-4-amino-3-hydroxy-S-phenylpentanoic acid, abbreviated AHPPA, phenylstatine, PS or phenylsta).

51. The presence of each of those designated peptides was verified by mass spectral analysis. It showed masses for the native form of 2021 and its Na+ adduct 2043 as well as the expected transition state analog masses of 2066, 2088 (Na+) and 2110. That mixture of native and transition state peptides has been abbreviated phenylstatine. Alz PheSta, Alz PS and Alz TS. Coupling this mixture of native and transition state β-amyloid peptides to maleimide activated Keyhole Limpet Hemocyanin created the first Alzheimer' s vaccine (that composite vaccine was abbreviated Alz-KLH or Alz-TS-KLH, indicating Alzheimer-Keyhole Limpet Hemocyanin and Alzheimer-Transition State-Keyhole Limpet Hemocyanin). (B001458-B001466).

52. The purification of the synthesized peptides was completed before December 2, 1997. The HPLC (high pressure liquid chromatography) analysis yielded peaks representing synthesized peptides of discrete masses, and the data also indicated that

the material in the late peak did not dissolve well in water and may have been an amyloid complex. In addition to the HPLC analysis, mass spectral analyses were also carried out in order to verify the identity of the peptides. (B001467-B001481).

53. Before December 2, 1997, I then went on to immunize four mice with the Alzheimer's peptides. Initially, the mice were vaccinated with 50 micrograms of Alz-TS-KLH in complete Freund's adjuvant (CFA) and then boosted with the same antigen in incomplete Freund's adjuvant (IFA) four months later. (B001482). The timing of the second boost is important for ensuring effective immune response in order to optimize the subsequent step of monoclonal antibody production.

54. Hybridoma cell fusions for the production of monoclonal antibodies were performed a month after the vaccination with IFA and several days after a subsequent i.v. booster injection with the antigen in PBS. This procedure involved isolating the spleen cells from immunized mice and carrying out somatic cell fusion of the immune cells with a highly proliferative line of myeloma cells in culture. At this point, I grew these hybridomas in culture and diluted the samples to isolate clonal colonies. Each colony, representing dividing cells derived from a single cell origin (clonal), was manually isolated, then the isolated colony was in turn grown separately to establish each hybridoma line. (B001483-B001493). This process typically takes several weeks to a few months.

55. Before December 2, 1997, I also worked on establishing additional β-amyloid peptides to be used as a vaccine. Assorted human β-amyloid peptides were synthesized, which were distinct from those made previously but were constructed using a similar random substitution strategy. Thus, this peptide synthesis was designed

to yield a mixture of eight peptides encompassing amino acids 35-43 of native human β-amyloid plus a cysteine for thioether coupling to an antigenic carrier. These peptides included the native human sequence (Met-Val-Gly-Gly-Val-Val-Ile-Ala-Thr) plus seven modified versions in which the valine and isoleucine residues were randomly substituted with a statine transition state analog. This peptide mixture was abbreviated AlzSta as opposed to the Alz-phenylstatine or AlzPS designation for the first peptide. The human amyloid β-peptide termed Alz 14-25 C-Y was designed both as a vaccine to generate antibodies and as a central region peptide that was labeled with radioactive iodine. (B001494-B001519).

56. In order to convert these peptides into usable vaccines, I then carried out chemical coupling of the peptides with an immune stimulant that acts as a carrier. The coupling of the Alz-statine peptide mixture synthesized to both maleimide activated Keyhole Limpet Hemocyanin (KLH) and maleimide activated Ovalbumin (OVA), which are commonly used carriers in the conjugation of peptides for antibody production, was conducted before December 2, 1997. This process created antigens for vaccination. The non-conjugated peptides were used for ELISA analysis of monoclonal antibodies. (B001520).

57. To generate an ascites which is a rich source of these different monoclonal antibodies directed against human β amyloid, immunization of mice using these antigen was carried out before December 2, 1997. Five mice were vaccinated with the AlzStaKLH peptides in CFA. The mice were then boosted with the same antigens in IFA four or five weeks later and finally an i.v. booster one month later with the antigen in PBS. (B001521-B001523).

58. I analyzed monoclonal antibodies generated using the β-amyloid peptide vaccine Alz-TS-KLH before December 2, 1997. I screened antibodies by ELISA assays. The spleen for the hybridoma fusion was obtained from one of the mice initially immunized. As predicted, this ELISA data proved that vaccination with the composite vaccine produced some antibodies that bound to native, human, full-length, β-amyloid, some that bound exclusively to the statine transition state peptide, and some that cross-reacted with both the native and transition state peptides. (B001524-B001556).

59. Before December 2, 1997, I carried out comparative analyses of the biodistribution of intravenously injected native, human, full-length, radioactive ^{125}I-β-amyloid in an untreated mouse versus a mouse that had been previously immunized with the ALZ Phenyl Statine composite vaccine. (B001557-B001558). This demonstrated that immunization of an animal with a human β-amyloid vaccine can effect the distribution of human β-amyloid in the body of an animal. Thus, it was shown that β-amyloid vaccination had the potential for a positive therapeutic impact on the Alzheimer's disease process.

60. I subsequently started preparing frozen stocks (liquid nitrogen) of early hybridoma clones that produced Alzheimer's disease-related antibodies directed against human β-amyloid, including Alz phenyl Statine clones, which were derived from animals that had been immunized with the Alz-Phenylstatine vaccine. Similarly, frozen samples from the Alz-Statine clones that were derived from animals that had been immunized with the Alz-Sta peptide vaccine were also prepared before December 2, 1997. (B001559-B001584).

61. Native, human, full-length, radioactive ^{125}I-β-amyloid (abbreviated ^{125}I-Alz peptide) was purified by HPLC before December 2, 1997. (B001585).

62. Subsequently, I performed before December 2, 1997, PEG assays and measured the direct binding of native, human, full-length, radioactive β-amyloid ("^{125}I-Alz_{1-45}") to a vast array of monoclonal anti-phenylstatine antibodies. These antibodies were obtained from a hybridoma fusion that used the spleen of a mouse that was immunized with the first vaccine synthesized. (B001586-B001608).

63. Before December 2, 1997, I began preparing a new set of hybridomas from the spleen of an animal immunized with the AlzStaKLH composite peptide vaccine (B001609-B001663). Subsequent ELISA analyses followed.

64. Also before December 2, 1997, I anticipated the need for an animal model to test the effectiveness of this vaccine therapy for Alzheimer's disease. I read that such a model was freely available to researchers (Hsiao, K., P. Chapman, S. Nilsen, C. Eckman, Y. Harigaya, S. Younkin, F. Yang, and G. Cole. 1996. Correlative memory deficits, Aβ elevation, and amyloid plaques in transgenic mice. Science 274:99-102). I telephoned Dr. Hsiao about the procedure for obtaining breeder animals needed to begin a colony of Tg2576 transgenic Alzheimer' s mice. After some time, I received an initial response from Dr. Hsiao to the request that I had previously made for obtaining Alzheimer's animal model. More specific correspondence regarding the delivery and handling of the Tg2576 mice occurred. (B001731-B001733).

65. Before December 2, 1997, our animal facility at BBRI finally received the delivery of the requested transgenic mice, Tg2576. The 2 female Tg2576 mice were used as 'founders' to begin our transgenic Alzheimer's mouse colony. About 7 months

was required to establish a viable Tg2576 colony of sufficient size to proceed with active and passive immunization experiments. (B001734).

66. I subsequently laid out a schedule for and began passive immunization of Tg2576 mice using the 5A11 monoclonal antibody. This antibody binds to human β-amyloid and was produced from a hybridoma fusion that used the spleen of a mouse that was immunized with the first vaccine synthesized. (B001800).

67. Before December 2, 1997, 5 normal mice were pristane primed and then on March 20, were injected with AlzSta clones 11E9, 5C7 and AlzPS clone 5A11. This procedure generates an ascites which is a rich source of these different monoclonal antibodies directed against human β-amyloid. (B001521-B001523).

68. Before December 2, 1997, I conducted a biodistribution study of radioactive human β-amyloid $A\beta_{1\text{-}40}$ in mice immunized with β-amyloid versus control mice immunized with KLH. (B001664-B001666). I also carried out related experiments measuring the blood levels of radioactive human β-amyloid after injection into mice passively and actively immunized with human β-amyloid agents. (B001667-B001674).

69. Experimental activities during the period between late 1997 to late 1998 may be characterized as the transitional time, in which earlier biochemical and cellular-based data culminated into in vivo animal-based applications. Accordingly, in addition to my continued effort to prepare for and submit grant applications, much of my time during this period was spent on obtaining and maintaining appropriate mouse colonies, ensuring for compliance with the institutional animal use protocols, and learning new animal-based laboratory techniques involved in the process.

70. In particular, I focused on in vivo experiments pertaining to vaccines using transgenic mice. More specifically, the Alzheimer technology I developed during this time period encompassed active as well as passive immunization for the treatment and/or prevention of Alzheimer's disease. The active vaccines I developed included both naturally occurring β-amyloid peptides as vaccines to elicit conventional (binding) antibodies and transition state analog β-amyloid peptide vaccines to elicit catalytic antibodies that bind and hydrolytically cleave β-amyloid into harmless fragments. For passive vaccination I initially injected either conventional binding antibodies directed against naturally occurring β-amyloid peptides or catalytic antibodies directed against transition state analog β-amyloid peptides.

71. Work on these basic concepts (active binding vaccines, passive binding antibodies, active catalytic vaccines and passive catalytic antibodies) was simultaneously initiated before December 2, 1997 when I synthesized the first multiplex peptide cocktail. I designed that preparation to contain both natural and transition state analog β-amyloid peptides. I proceeded to use that formulation as an active vaccine in normal and transgenic mice so that both conventional and catalytic antibodies would be induced. I subsequently derived both conventional and catalytic monoclonal antibodies from normal immunized mice and utilized those anti-β-amyloid antibodies as passive vaccines.

72. While the active and passive immunization of the transgenic mice described above was progressing, I continued to simultaneously develop three closely related areas: 1) catalytic antibodies designed to cleave and inactivate β-

amyloid, 2) vectorized bispecific antibodies designed to deliver anti-β-amyloid antibodies across the blood-brain barrier and into the central nervous system and 3) additional new vaccines to cure/prevent Alzheimer's disease. These studies and the corresponding data generated are best summarized in the six grant applications Bates numbered B00269A-, B000270- and its resubmission B000476-, B000313-, B000340- B000366-. Furthermore, yet other areas of vaccine research were ongoing. This additional work is documented in B001565, B001564, B001566, B001675, B00673, B000749, B001804 and B001675-B001682.

73. Regarding evidence of activities conducted during this time period, much of the relevant data was obtained with and is stored on a computer-based molecular imager workstation. That data is mostly in the form of dated digital image files. This collection of data is provided in two groups. One set has been printed directly from the workstation that automatically incorporates the date created. This set is identified by Bates numbers B001816-B001939, and includes both a listing of the files (B001816-B00I845) and the image files themselves (black and white) (B001846-B001939). This first set contains the computer file listing for all of the Alzheimer's vaccine related material found on the molecular imager workstation that was created between 11/25/97-2/26/03. The second set contains related data (color images) that was printed from the computer and has been hand dated according to the associated file listing. In this second set the file listing is identified by B001940-B001941, and the images are identified by B001942-B002001.

74. The molecular imager workstation is used to collect and visualize quantitative images of radioactive or light-emitting samples. Briefly, during this time period I used this system to collect data for the following categories of Alzheimer's vaccine research:

1) Catalytic Antibodies: detect and measure the ability of catalytic monoclonal antibodies to cleave radioactive β-amyloid into smaller fragments;
2) Passage of Anti-β-Amyloid Antibodies Across the Blood-Brain Barrier: detect, visualize and measure the ability of radioactive vectorized bispecific antibodies to enter the brain of living normal and transgenic mice over the course of time following passive immunization;
3) Genetically Engineered β-Amyloid Fusion Vaccines: determine the size of fluorescently tagged genetically engineered β-amyloid DNA that we used to make new fusion vaccines for active immunization;
4) Interaction Between β-Amyloid and Anti-β-Amyloid Antibodies: follow the migration of radioactive or fluorescent β-amyloid peptides on gels and measure their binding to the different monoclonal anti-beta amyloid antibodies; and
5) Assorted additional experiments, files, grant material, etc. related to my Alzheimer's vaccine research.

75. The image data in the first and second set of documents indicates that there was consistent development in areas 1-4 from December 1997 through the end of April 1998, and then more in June and August 1998, when a series of vaccine experiments was completed and the animals were ready to be analyzed histologically.

76. To analyze and document histological evidence of these experiments, the brain tissues were collected and analyzed in September 1998. I looked at the multiple microscope slides prepared by the SERI Morphology Group during this period, including slides having the following designations: 1363 Brown AA92 (-), 1363 AA92 Black +, 9/17/98. 1391 Alz KLH, 1072 10μ, 9/17/98. 1095 9-10-96 Treated (this is smudged and is probably 1095 9-10-98 Treated). 1391 Control KLH, Normal 12μ 9/17/98. These designations correlate with document B01803, and can probably be correlated with the treatment schedules on the mouse cage cards and the mouse inventory sheets.

77. Much of the work described above is summarized in my grant applications, for example, a PHS 398 form submitted to NIH in October 1998, entitled "Cerebral Delivery of Vectorized Anti-β-Amyloid Antibody" (Bates No. 000476-). Included in the grant application is a set of preliminary data that I had collected up to this point, since I first conceived the invention in 1996. In particular, much of the crucial data involving in vivo systems, i.e., mouse models, came from my experiments conducted during the time period at issue. These include preliminary data regarding: Transcytosis of bispecific antibody into the brain (B000505-506); Monitoring the brain distribution of bispecific antibody in live mice (B000506-507); Distribution of plaque development in transgenic mice (B000507). Each is briefly discussed below.

78. It is always a challenge to deliver a pharmaceutical composition to the brain because of the existence of the "blood brain barrier." The blood brain barrier is a membrane that controls the passage of substances from the blood into

the central nervous system. It is a physical barrier between the local blood vessels and most parts of the central nervous system and stops many substances from traveling across it, thereby presenting technical obstacles in therapies associated with the brain of an animal.

79. To overcome this barrier, I sought to "vectorize" the antibody, that is, to couple it to another antibody against a protein that is present in the inner wall of the blood vessel so that it promotes the attachment of the compound to the wall, and subsequent "uptake" or crossing of the barrier. The "transcytosis of bispecific antibody" experiment demonstrated that the bispecific antibody could indeed cross the blood brain barrier and be actively delivered into the brain of normal mice.

80. This was determined by a set of experiments called capillary depletion method. In short, the significance is that when the labeled vectorized bispecific antibody is injected i.v. into mice, it was able to attach to appropriate receptors on the luminal side of the vessel then cross the blood brain barrier by the process of transcytosis. This allowed the delivery of the bispecific antibody in the brain, where the Aβ is localized.

81. Once I obtained the data showing that the bispecific antibody is indeed capable of crossing the blood brain barrier and be delivered into the brain of normal mice, I then conducted experiments to monitor the brain distribution of bispecific antibody using live mice. Series of data of brain images were sequentially collected at various time points after the i.v. administration of the bispecific antibody.

82. Having established that the vectorized bispecific antibody that binds to Aβ aggregates was capable of being delivered to the cite of interest (the brain) in normal mice, I then sought to test its effectiveness in the Tg2576 transgenic animals, which exert pathological features that are a murine equivalent of human AD. Indeed, these transgenic animals form Aβ plaques, which are a hallmark of the disease in humans.

83. I invested much time and effort during this time in establishing a transgenic mouse colony so that I would have a sufficient number of mice to be used in testing my reagents. By this time, I was able to learn and perform some histology, e.g., process and cut brain sections for immunocytochemical and thioflavin S detection of amyloid plaques in these mice. And my transgenic colony had just been expanded enough for performing experimental studies on the cerebral delivery of anti- Aβ antibodies. I referred to this work in my grant applications filed October 21, 1997 [Ex. R] and December 11, 1997 [Ex. S], which reflect the laboratory work that had been performed before December 2, 1997.

84. As can be seen from the attached chart [Ex. N] and the documents it cites [e.g., B001760-1795; 001807-1812; 001796; 001800; 000744-745; 000746-748; 000754-756], I continued to work in the lab on a β-amyloid Alzheimer's vaccine. For example, in Cage ID AA-92, the white and black mice were immunized with Alz-KLH in CFA on September 12, 1998 and boosted on October 24, 1998 while the brown mouse got KLH control immunizations on September 13, 1998 and October 24, 1998. These experiments were designed to test the injection of Alzheimer' s vaccines (active immunization) for therapeutic effect. The comments column reading DOA 9/16/97

2Bk=5A11 and the other which reads "DOA 9/16/97 Br=5A11" designate animals that were treated with the purified 5A11 monoclonal antibody. These kinds of experiments were designed to test the injection of anti-Aβ (passive immunization) for therapeutic effect. The 5A11 antibody binds human β-amyloid and was obtained from the hybridoma fusion which used one of the mice initially immunized. (B001760-B001799).

85. Similarly, on August 11, 1998 and September 22, 1998, I requested histological analyses from SERI Morphology service. Frozen brain sections were cut for the Tg2576 mice. We stained and examined these sections microscopically for the presence of β-amyloid plaques. A sheet dated September 22, 1998 lists some of the microscope slides with brain sections from Tg2576 mice that were treated with the 5A11 anti-human β-amyloid monoclonal antibody passive vaccine. (B001801-B001803).

86. On September 12, 1998, I outlined an immunization schedule and vaccine components, and for immunizing the ALZ Tg2576 mice. As indicated the vaccine was comprised of a cocktail of three different human native β amyloid peptide-KLH antigens. NH2-terminal-KLH is amino acids 1-16 of human β amyloid coupled to KLH via a cysteine-mediated thioether linkage. Middle-peptide-KLH is amino acids 10-25of human β-amyloid coupled to KLH via a cysteine-mediated thioether linkage. COOH-terminal-KLH is amino acids 35-43 of human β amyloid coupled to KLH via a cysteine-mediated thioether linkage. The brains of these mice were removed for sectioning and analysis on October 23, 1999 (B001804-B001812).

87. Invention Disclosures

88. I submitted invention disclosures for my β-amyloid vaccine to the BBRI on November 22, 1996 [Ex. AF], November 4, 1997 [Ex. AG], and December 7, 1998. [Ex. AH] The BBRI declined to file a patent application the first two times, requesting additional data to support the patent application. The third time, it was judged that I had developed sufficient data, thanks in part to the small SBIR grant I received in 1998. Accordingly, the BBRI decided to file a patent application at that time.

89. I discussed the patent application with BBRI's outside patent agent on February 23, 1999. She requested some further information, which I provided her on February 26, 1999. [Ex. AI]. On March 2, 1999, she sent me a facsimile requesting electronic copies of some of my grant applications relating to my β-amyloid vaccine. [Ex. AJ]. I provided that information to her, and also provided her with additional data on March 5, 1999.

90. I reviewed several draft patent applications from BBRI's patent agent and provided comments, revisions, and any requested data. I understand that she filed the application on June 22,1998 as Patent Application No. 60/050,388.

The above statements are made with the knowledge that willful false statements may be punishable as provided by 35 U.S.C. 25 and 18 U.S.C. 1001.

Victor A. Raso

VICTOR A. RASO, Ph.D.

AFFIDAVIT OF VLADIMIR VOLLOCH

I, Vladimir Volloch, depose and swear as follows:

1. I was employed by Boston Biomedical Research Institute, then located at 20 Staniford Street, Boston Massachusetts 02114 ("BBRI"), between January 1, 1983 and March 31, 1998, and am currently employed by Tufts University.

2. On May 2, 1996, beginning at 12:00 p.m., I presented a lecture at BBRI entitled "Alzheimer Disease - A Minireview."

3. On May 2, 1996, shortly after the lecture, I had a conversation with Victor Raso. I recall that Dr. Raso discussed his ideas on the development of both active and passive vaccines for curing or preventing Alzheimer's disease by using beta-amyloid as an antigen. He mentioned using native beta-amyloid antigens to elicit antibodies that would tightly bind beta-amyloid and sequester it in the body. I recall that he also proposed the use of transition state beta-amyloid antigens that would generate catalytic antibodies that could irreversibly cleave beta-amyloid into harmless fragments.

Dated at Medford, Massachusetts, this 12 day of September, 2003.

By: [signature]
Vladimir Volloch

COMMONWEALTH OF MASSACHUSETTS
COUNTY OF MIDDLESEX, SS.

On this 12th day of September, 2003, personally appeared Vladimir Volloch, known to me, or satisfactorily proven to be the person who is the signatory to the foregoing, and made oath that the forgoing instrument, subscribed by him/her, is true.

Before me,

Carolyn Resendes
Notary Public
My Commission Expires: 10/4/09

BTV/250066.1

AFFIDAVIT OF HENRY PAULUS

I, Henry Paulus, depose and swear as follows:

1. I am an employee of Boston Biomedical Research Institute, 69 Grove Street, Watertown, Massachusetts ("BBRI"), and have been employed by BBRI since November 6, 1975.

2. On May 2, 1996, beginning at 12:00 p.m., I attended a lecture at BBRI given by Vladimir Volloch. The lecture was entitled "Alzheimer Disease - A Minireview."

3. On May 2, 1996, shortly after the lecture, I had a conversation with Victor Raso. I recall that Dr. Raso discussed his ideas on the development of both active and passive vaccines for curing or preventing Alzheimer's disease by using beta-amyloid as an antigen. He mentioned using native beta-amyloid antigens to elicit antibodies that would tightly bind beta-amyloid and sequester it in the body. I recall that he also proposed the use of transition state beta-amyloid antigens that would generate catalytic antibodies that could irreversibly cleave beta-amyloid into harmless fragments.

Dated at Watertown, Massachusetts, this 12th day of September, 2003.

By: [signature]
Henry Paulus

COMMONWEALTH OF MASSACHUSETTS
COUNTY OF MIDDLESEX, SS.

On this 12th day of September, 2003, personally appeared Henry Paulus, known to me, or satisfactorily proven to be the person who is the signatory to the foregoing, and made oath that the forgoing instrument, subscribed by him/her, is true.

Before me,

Dorothy Synge [signature]
Notary Public
My Commission Expires: 10/4/07

BTV/250030.1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):	Victor Raso
Application No.:	09/992,994
Filing Date:	November 6, 2001
Title:	IMMUNOLOGICAL CONTROL OF β-AMYLOID LEVELS *IN VIVO*
Art Unit:	1652
Examiner:	Patterson, C.

DECLARATION OF HENRY PAULUS, PH.D.

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Henry Paulus, do hereby declare and say:

1. I am a Senior Scientist, with tenure, at the Boston Biomedical Research Institute (BBRI). I joined BBRI in 1975 as a Senior Scientist and Director of the Department of Metabolic Regulation (1975-1992) and have served as Deputy Executive Director (1983-1985), Executive Director (1985-1987) and Director (2001-2003). I also served as Deputy Director (1990-2001).

2. I have also served since 1969 as an Associate Professor in the Department of Biological Chemistry and Molecular Pharmacology at Harvard Medical School. I have held several positions in the Department of Biological Chemistry at Harvard Medical School, including Instructor (1961-1964), Associate ~~Professor~~ (1964-1967), and

HP
3.7.07

Assistant Professor (1967-1969). I received a B.A. and B.S. in (1957) and a Ph.D. (1959) from the University of Chicago. I was also a Postdoctoral Fellow in the University Chemical Laboratory at Cambridge University, England (1959-1960) and in the Department of Chemistry at Harvard University (1960-1961).

3. I attended a seminar by Dr. Vladimir Volloch at BBRI on May 2, 1996 beginning at 12:00 p.m. [Ex. A]. Vic also attended the lecture. After the lecture, Vic spoke to Vladimir and me about his idea to use β-amyloid as an antigen to generate active and passive vaccines for Alzheimer's Disease. Vic discussed using various native β-amyloid antigens to elicit antibodies that would tightly bind β-amyloid in the body and sequester it. He also proposed the use of transition state β-amyloid antigens to generate catalytic antibodies that would irreversibly cleave β-amyloid into harmless fragments.

4. The BBRI is an independent, not-for-profit biomedical research institute. As such, it is dependent upon federal grants, principally from the National Institutes of Health (NIH), for its revenues. The typical NIH grant received by BBRI provides the BBRI with direct costs for the research project (salaries, supplies, etc.) and, indirect costs (overhead costs, such as light, heat, administrative services, etc.) [Ex. B at p. 7]. With few exceptions, the salaries of BBRI faculty are paid by such grants as direct costs. BBRI faculty are expected to be self-funded, generating their income via such grants.

5. While the BBRI depends upon grants to pay faculty salaries as direct costs, such grants are also very important to the BBRI because they fund the overall budget as indirect costs. Otherwise, the grant would not reimburse BBRI for the cost of the entire

project [Ex. B at p. 4]. The Director of BBRI or, in his/her absence, the Deputy Director is responsible for all financial matters [Ex. B at p. 13]. From my service as Deputy Director, as well as my other positions at BBRI, I am particularly aware of the drawbacks of grants that do not include indirect costs. Each time the BBRI accepts such a grant, it is essentially losing money.

6. Because BBRI faculty are expected to be self-funded, they are expected to generate new grants regularly. BBRI faculty hold appointments of three, four, and five years for Scientists, Principal Scientists, and Senior Scientists, respectively [Ex. C at p. B-06a]. Reappointment to an additional term is dependent upon four criteria. The primary criteria is grant support [Ex. C at p. B-03]. In certain cases, however, BBRI will award tenure to faculty members, resulting in a permanent appointment [Ex. D at p. 4].

7. The BBRI provides temporary funding to faculty members who are thought to be likely to regain funding [Ex. E at p. A-22]. Bridge Support is available for only one year, other than in "exceptional circumstances." [Ex. C at p. E-06]. The primary criteria for determining whether such an exceptional circumstance exists is the probability of funding [Ex. C at p. E-06]. As Deputy Director, I was a member of the Committee on Research (COR), which makes recommendations regarding Bridge Support, in 1996 [Ex. F at p. 1].

8. Vic was already on Bridge Support when the BBRI formalized its policy. He requested a second year of Bridge Support in late 1996. In support of his request, he indicated that he was actively working on five different grant applications [Ex. G at p. 1]. Although he did not appear to meet the new criteria, the Committee approved further Bridge Support, although for only 8 months, not a full year [Ex. F at p. 2].

9. Vic continued to seek funding. His many and repeated efforts were not successful, however. Vic also tried to obtain other sources of funding, seeking Small Business Innovation Research Grants (SBIR), as well as grants from the Alzheimer's Association. Having failed to get the typical NIH grants, Vic tried these sources, even though he knew the BBRI was not terribly interested in such grants because such grants do not provide full indirect costs. Indeed, Vic was a member of the COR that voted to contingently grant further bridge support to a faculty member [Ex. F at p. 1]. That vote was contingent on the faculty member obtaining NIH funding. The COR specifically stated that "an Alzheimer's [Association] grant would not be enough." [Ex. F at p. 1].

10. Vic continued to fail to obtain funding. Therefore, it appeared likely that he would not be reappointed as a Senior Scientist when his term ran out in 1999.

11. Moreover, BBRI allocates laboratory space based upon funding and utilization levels [Ex. C at p. B-06a]. Besides facing the likely prospect of not being reappointed, Vic was at risk of losing some or all of his laboratory space. This did happen to Vic so that, by late 1999, he had lost all his space except for one-half a laboratory bench and his office.

12. Although Vic was not meeting BBRI's requirements, I always enjoyed working with him and kept apprised of his activities. At this time, Vic was constantly working on grant applications in an attempt to obtain a salary to support himself and his family, as to retain his position on the faculty, and to retain his laboratory space. From January 1997 until August 1999, he prepared and submitted 16 grant applications. He asked me

for my comments on several of these applications. These 16 grant applications all concerned Alzheimer's Disease, particularly the use of antibodies to β-amyloid:

Date	Type of Grant (Form)	Title	Bates Number
01/15/1997	NIH (PHS 398)	AMYLOID β PEPTIDE EQUILIBRIA IN "ALZHEIMER'S MICE"	B000030
08/29/1997	NIH; SBIR (6246-1)	IMMUNOTHERAPY OF ALZHEIMER'S DISEASE	B000093
10/21/1997	NIH (PHS 398)	AMYLOID β PEPTIDE EQUILIBRIA IN "ALZHEIMER'S MICE"	B000223
12/11/1997	NIH; SBIR (PHS 6246-1)	CEREBRAL ANTIBODY DELIVERY TO TREAT ALZHEIMER'S DISEASE	B00269A-269Y
01/26/1998	NIH (PHS 398)	CEREBRAL DELIVERY OF VECTORIZED ANTI-β-AMYLOID ANTIBODY	B000270-312
04/13/1998	NIH; SBIR (PHS 6246-1)	CATALYTIC ANTIBODIES TO INACTIVATE β-AMYLOID	B000313-339
05/20/1998	NIH (PHS 398)	VACCINE TO MODULATE SYSTEMIC β-AMYLOID LEVELS	B000340-365
05/27/1998	NIH (PHS 398)	PROBE TO VISUALIZE CEREBRAL β-AMYLOID PLAQUES	B000366-392
09/01/1998	Alzheimer's Association (KRS)	VACCINE TO MODULATE (SYSTEMIC) BETA-AMYLOID LEVELS	B000432-449
09/03/1998	Alzheimer's Association (KRS)	CEREBRAL DELIVERY OF VECTORIZED ANTI-β-AMYLOID ANTIBODY	B000393-413
09/04/1998	Alzheimer's Association (KRS)	CATALYTIC ANTIBODIES TO INACTIVATE β-AMYLOID	B000414-431
10/11/1998	NIH (PHS 398)	CEREBRAL DELIVERY OF VECTORIZED ANTI-BETA-AMYLOID ANTIBODY	B000476-523
01/19/1999	NIH (PHS 398)	IMMUNOTHERAPEUTIC AGENTS TO TREAT ALZHEIMER'S DISEASE	B000141
01/27/1999	NIH (PHS 398)	NOVEL TRANSITION STATE PEPTIDE ANALOG ANTIGENS	B000524-569
04/12/1999	NIH; SBIR (PHS 6246-1)	BIPHASIC PROBE TO VISUALIZE INTRACELLULAR BETA-AMYLOID	B000450
08/13/1999	NIH; SBIR II (PHS 6246-2)	IMMUNOTHERAPY OF ALZHEIMER'S DISEASE	B000187

13. In my experience at BBRI, this is a tremendous number of grant applications to prepare and submit in about 2 ½ years, especially for someone working largely on their own. The submission of a grant application to is a major undertaking, which can take

three to six months to write. For example, the Grant Application Writer's Handbook, published in 1995 by Liane Reif-Lehrer (Jones & Bartlett, publishers) describes the various steps in grant application writing and the amount of time these typically take, several weeks to several months for preparation of an application alone (*excluding* the time it requires to accumulate the preliminary data on which the grant application is based) [Ex. H]. Vic was able to submit so many grant applications during this period because he was working on them non-stop.

I understand that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified patent.

Henry Paulus, Ph.D.

Date: 7 March 2007

CBMG Journal Club

Thursday

May 2, 1996

[Please note change of date]

Topic:

Alzheimer Disease - A Minireview

Speaker:

Vladimir Volloch

Second Floor Conference Room

12:00 noon

B000001

Approved by
Board of Trustees
1996

BOSTON BIOMEDICAL RESEARCH INSTITUTE
Program for the Future

STRATEGIC PLAN OUTLINE

SCIENTIFIC PROGRAM

I. **Faculty Development**
   - A. New Faculty Recruitment
   - B. Bridge Support
   - C. Fringe Funding
   - D. Unreimbursed Indirect Costs
   - E. Scientific Support Initiative

II. **Capital Equipment**
   - A. Structural Biology Facility
   - B. Protein Expression Facility

III. **Space**

BUSINESS PROGRAM

I. **Revenues**
   - A. Grants (Faculty-Initiated)
     1. Federal
     2. Other Agencies (Non-Federal)
     3. Pharmaceutical/Industrial Collaborations
   - B. Development/Public Relations
   - C. Investments
   - D. Technology Transfer

II. Expenses
   - A. Directs
   - B. Indirects

BOSTON BIOMEDICAL RESEARCH INSTITUTE
Program for the Future

STRATEGIC PLAN

SCIENTIFIC PROGRAM

I. Faculty Development

The long-range scientific strategy is to build upon our current reputation as the center for *muscle research* in the Boston community by not only further developing this area of investigation but also developing two additional interacting areas of research excellence. These two additional areas will be *cellular communication* and *cellular growth*. By developing strong basic science programs in these three overlapping specialities, we will have the necessary depth and scope to be able to make major contributions to health issues such as stroke, heart failure, and hypertension.

In the area of *muscle research*, additional scientists in the field of nonmuscle motility should be recruited. The successful recruitment of scientists working on novel molecular motors and on cytoskeletal-matrix interactions in nonmuscle cells will allow the Institute to apply its half-century of expertise in contractile proteins to much broader questions such as the "crawling" of cancer cells during metastasis and the trafficking of messages within cells. Thus, these scientists should be able to interact not only with the muscle group, but also the cell growth group and the cell communication group.

In the area of *cell communication*, the Institute already has considerable expertise in the area of transport proteins, and the recruitment of a channel person with patch clamp and perhaps channel cloning abilities would allow focused research on transmembrane communication in a much broader sense and in a manner that could greatly enhance investigations into motility and cell growth. Additionally, the recruitment of molecular biologists working on kinases and signalling cascades in both differentiated and proliferative smooth muscle cells would strengthen this focus group and assure interactions with the muscle and cell growth groups.

In the area of *cell growth*, the recruitment of a cell-cycle person or a growth factor person could greatly increase relevance of ongoing research into DNA and RNA replication mechanisms and would enhance the possible collaborative interactions of the muscle and cell communication groups.

A. New Faculty Recruitment

In the current era of financial crisis in biomedical research, fewer and fewer young graduates are entering basic research. This has created an intense competition among academic institutions for the best and brightest scientific graduates. At the same time, given the current shortage of federal funds for support of scientific

research, it is exceedingly difficult for the new scientist to become established as a recognized expert in a field of investigation and thus successfully compete for federal funds. Only the best of the young scientists do succeed and, on average, three to five years appears to be required for the new individual to become entirely self supporting. For this reason, the most competitive research centers are now offering five year packages of guaranteed support during the recruitment process. BBRI currently has the financial means to offer support for only two to three years, thus decreasing our competitiveness in recruiting new faculty and also, in some instances, forcing us to terminate promising young individuals before we have the opportunity to see any return on our initial investment. Supplementation of current resources by the creation of named Young Scientist Awards would greatly increase our chances of recruiting and retaining top investigators.

Estimated costs for recruiting two new Principal Investigators each year are: PI's salary $60K x 2 = $120K; Tech $25K x 2 = $50K; Salaries = $170K; x2.6 indirects and fringes = $442K; Start-up = $40K; Supplies $10K x 2 = 20K; Relocation and remodeling = $30K. Total: $532K. Current estimates indicate that we should recruit two scientists per year in order to optimize grant income and maintain the vitality of the scientific programs.

B. Bridge Support

An equally urgent need is bridge funding of established investigators to cover the inevitable intervals when federal funding is delayed. A general goal will be to decrease the excessive dependence on NIH and similar sources for funding. Although federal funds will undoubtedly remain the main support for basic research, efforts should be made to create a "mixed portfolio" including industrial contracts, pharmaceutical and clinical collaborations, foundation support, and endowed, named Senior Scientist Awards. The interest on BBRI's endowment should be a major source for bridge funding, hence the importance of maintaining and increasing the current principal. Current experience indicates that providing bridge support for investigators averages $225,000 annually.

We currently have the resources to provide support equal to the Principal Investigator's salary for one year. This policy inevitably results in loss of valuable intellectual capital. The long range goal is to develop resources to provide up to two years of support for the Principal Investigator and one member of the research team.

C. Fringe Funding

Currently, most of a Principal Investigator's time is focused on acquiring and maintaining NIH support. It is generally acknowledged that the NIH will only support ideas that are clearly documented to be entirely "feasible" by the presentation of considerable "preliminary data" and demonstrating that pilot studies have already been successfully performed. Truly novel and creative ideas cannot be supported by this mechanism and as a result the true potential of the

"intellectual capital" of the Institute is not being tapped. Hence there is a need for a funding mechanism to support ideas on the "fringe" of established concepts and pilot studies that will provide preliminary data that will eventually lead to NIH support. No funds are budgeted in this five-year period for this program, but fundraising efforts by the Development team could be focused on creating such a program.

D. Unreimbursed Indirect Costs

There are a number of agencies that provide grant funding that does not include indirect costs or, in other words, grants that do not reimburse the Institute for the cost of the entire project. Many of these grants, however, are highly prestigious and it is greatly to BBRI's advantage to have our faculty receive such awards. It is necessary, therefore, for BBRI to anticipate allocating funds to cover the unreimbursed indirect costs associated with these research projects. This currently involves the use of about $150,000 of Institute funds annually for sharing incompletely reimbursed indirect costs.

E. Scientific Support Initiative

It is also imperative that BBRI initiate an outreach program to attract the necessary support resources for BBRI's scientists, e.g. students from local colleges and universities; post doctoral fellows; M.D.s in specialty training programs; visiting scientists; etc. BBRI offers a unique and ideal environment in which these talented young people can begin to explore or continue to build a career in basic research and our scientists will benefit from the additional research support as much as these individuals will benefit from the experience and the expertise at BBRI. It is equally important, however, that this initiative be implemented and maintained with minimal cost to the Institute.

II. Capital Equipment

A. Structural Biology Facility (attached)

B. Protein Expression Facility

Certain molecular biology techniques have now been sufficiently established to have become routine. In order to promote efficient utilization of BBRI resources and to capitalize on the collaborative nature of the Institute, efforts will be made to prevent the duplication of facilities that can be centalized and shared.

There is an immediate need for expression of proteins in essentially all BBRI laboratories. Rather than have each investigator independently devote personnel and time to a routine procedure, the Institute will set up a shared Protein Expression Facility. A Core Leader--a Principal Investigator-- will contribute 10% of his/her time to supervise the facility. A full-time research technician will be responsible for

day-to-day operations. Two-four large capacity incubaters/shakers and two laminar flow hoods will be located in a small, self-contained laboratory. The core will be responsible for producing proteins primarily by using the baculovirus expression system, including keeping the virus stock, making the constructs, maintaining insect cell cultures, performing co-transfectionand scaled-up production of proteins.

Funding for the core facility will be sought as part of the Smooth Muscle Program Project Grant (PPG), a competitive renewal grant to the NIH, and therefore will require minimal institutional funds.

III. Space

The current space available at BBRI allows the recruitment of only two additional investigators with rather modest space needs. Improving the air circulation and lighting in the basement and rearranging the existing space can optimize its use but, if the Institute is truly to be able to grow in the next millennium, some plan of action will have to be created for additional space.

BBRI still benefits from the lease negotiated with Schepens 25 years ago. In effect, BBRI does not pay for floor space itself but shares in the operating costs as outlined above. Operating costs are higher than those in a modern facility due to the inefficiencies inherent in an older facility. However, our per square foot cost of occupancy is considerably lower than at alternate sights.

The Institute has considered purchasing or leasing a different site for the facility. The lease does not allow BBRI to sublet space at Staniford Street, effectively limiting our sales market to Schepens Eye Research Institute. Although we know Schepens is seeking additional space, we feel it is unlikely that they will be prepared to offer us enough incentive to vacate. Economically, we can continue to enjoy the facility "rent free" for the next 25 years. It may be possible to renew the current lease but whether this would be a feasible, or desirable, option depends on the plans of the SERI, the condition of the building and the details of the new lease. Current strategic plans of the SERI appear to preclude extending the existing lease. It is to be recognized, however, that any plans to build onto the current building must be contingent on the eventual extension of the current lease.

Within the five-year Strategic Plan, we have factored in the following plans for the provision of adequate space.

<u>Rental:</u> Additional space could be rented in neighboring buildings. This could be done at a cost of $25/sq. ft. in the Charles River Plaza or the building at 50 Staniford Street. For fiscal year 1997, we are considering renting 1,000 square feet and relocating the Accounting and Development offices to this space. The current Accounting Office area would then be renovated to accomodate the X-ray crytallography equipment and program staff.

For fiscal year 1998, two plans have been developed. The first would be in anticipation of the natural attrition in any organization and would focus on recruiting new faculty to use the space that would be vacated in the process of attrition. The second plan would be to rent additional space in the Charles River Plaza. Again, rental of this space would be $25/square foot and it is likely we would be required to rent a minimum of 2,000 square feet initially, for a total of $50,000 annually. Renovation of that space would require roughly $120/sq. ft. If we assume a new investigator requires approximately 400 square feet, the initial construction cost for a laboratory would be $50,000. The close proximity would allow for use of shared equipment and interaction with other faculty.

Looking ahead, beyond the five-year Strategic Plan, the decision remains to be made regarding relocation of the entire Institute.

Relocation: The entire Institute could relocate. This would be by far the most costly and time consuming option but should be considered an option for long term planning. Alternative laboratory space is available in other areas of the city. For example, laboratory space in the Longwood Medical Area rents for about $55 per square foot ($22,000 annually for a 400 square foot lab), with the advantage of proximity to Harvard University personnel and affiliates.

One specific concern is the fact that although the Institute has the academic prominence to host major international conferences, there is currently no on-site auditorium of the necessary size for such a venture. If funds could be obtained, a named conference center would significantly enhance the mission of the Institute to discover and disseminate new biological knowledge.

BUSINESS PROGRAM

The remarkable track record of success of the faculty in bringing in 90% of expenses from grants is a credit to their level of scientific excellence. At the present time, the Institute receives roughly 90% of its operating budget from these grants, the majority of which come from the NIH. Given the current shortage of funds at the NIH, allowing only 9-15% of meritorious grants to be funded, a major goal over the next decade will be to create a more "mixed" portfolio, which will reduce the Institute's dependence on faculty-initiated grants from the NIH and similar agencies and increase the supporting income from long and short term industrial contracts, foundation support, private donations, and return on BBRI investments to approximately 40% of the budget.

I. Revenues

A. Grants (Faculty-Initiated)

1. Federal

The U.S. government is the single largest supporter of biomedical research in the world; in 1994, the U.S. spent about $33 billion for biomedical and related health research and development. The federal government provides approximately 80% of the monies spent each year for biomedical research at universities, medical schools, and not-for-profit research institutes. 87% of all federal support for biomedical research comes from funds allocated by Congress to the National Institutes of Health (NIH); the 1995 NIH budget was $11.3 billion.

The NIH continues to be BBRI's major source of grant funding. Occasionally, the Insitutute also obtains funding from the National Science Foundation (NSF). The typical Individual Investigator Initiated Research Project Grant, termed RO1, provides BBRI with direct research costs (salaries, research supplies, etc.) and indirect costs (i.e. those costs incurred by BBRI such as light, heat, administrative services, etc.).

The grant process at NIH is a long, stressful process for the scientist who has submitted a grant. The NIH receives grant applications 3 times a year. The applications are generally 30-50 page documents and require that all proposed experiments be accompanied by preliminary experiments to certify feasibility. Approximately 3-4 months after receipt, the grants are subjected to "peer review" by one of several "Study Sections", i.e. a committee of scientists from across the country that meets in Bethesda, MD. for 2-3 days to construct a relative ranking of the grants by merit. Currently, 4 of our faculty members serve on NIH Study Sections as nationally recognized experts in their fields.

The study section assigns a "priority score" from 1.00 to 5.00 to each grant--with 1.00 being most meritorious and 5.00 being least meritorious. Recently, in an effort to decrease administrative cost and time, a "triage" process has been implemented and grants that are evaluated to be in the worse half (generally scores of 2.5 and larger) do not receive full review by the committee or a score.

When the study section completes its evaluations, the scores are "percentiled" against past scores recommended by that individual study section. Only percentiles are compared between study sections, in order to normalize for differences in the generosity or toughness of scientists on the individual study sections.

Three to four months after the study sections complete their work, the grants receive a second level of review by the Councils of the NIH. Each Council will set a "payline". Current paylines are ranging from 9-15%. Often a grant is borderline and, also because of political uncertainties, the Principal Investigator may not know for sure whether he or she is funded until shortly before the start date of the grant, 3-4 months after Council meets. Thus there is significant lag (generally 9 months) between submission and funding of successful grants.

Because of the shortage of funding for excellent grants, most investigators expect to need to revise their applications at least once, adding another 9 months to the delay in funding. Current efforts are being made to try to submit renewal applications for funding one year early in an effort to keep programs (and personnel) continuous.

2. Other Agencies (Non-Federal)

There are several private national foundations to which investigators can apply for support. However, these sources require that the research be disease-oriented. Also, these grants are relatively small in dollar amount, and they do not provide continuous support of ongoing investigations. These smaller grants can be very useful, however, in adding needed personnel to a project or in providing support of an ongoing project while waiting for NIH funding to be awarded.

The Institute has received, and continues to receive, generous support from the Muscular Dystrophy Association for its work on muscle disease, from the American Heart Association for its work on stroke, heart failure and hypertension, and from the American Cancer Association for its work on mechanisms of cell growth.

3. Pharmaceutical/Industrial Collaborations

Increasing the revenue from industrial collaborations is a longterm goal; therefore, revenues from this initiative are not included in the five-year Strategic Plan. A task force should be established to acquire information on companies that may have long range goals or specific ongoing programs that mesh well with the goals and expertise of the Institute. This group should plan the initiation of contacts with the appropriate companies. The goals will be: (1) to initiate an "intimate relationship" with 1 or 2 major pharmaceutical firms, allowing them to have a "window" on evolving, state of the art, basic science in return for liscensing rights; (2) to initiate individual scientific collaboration between Industry and the Institute that may result in sponsored research; (3) to obtain named sponsorship for seminar programs; (4) to establish Sabbatical/Internship Programs that will allow industrial scientists and Institute scientists to learn specialized technologies and perform collaborative studies.

A joint venture with the Beth Israel Hospital has led to the establishment of the Boston Collaborative Heart Failure Center, encompassing a number of specific collaborative "discovery" research projects aimed at the cure of heart failure, through patient testing and multi-center clinical trial coordination of new potential therapies and diagnostic tools. Significant corporate support for the Center is being actively recruited.

Long term contracts with pharmaceutical companies may be of considerable benefit in providing financial stability, intellectual input, and access to new experimental tools. However, because of concerns regarding academic freedom, co-existing NIH

regulations, and legal complications, considerable caution should be applied before entering into such ventures.

B. Development/Public Relations

There are basically 3 sources of revenue for charitable contributions to BBRI:

REVENUE SOURCES

Individuals
Foundations
Corporations

Programs need to be strengthened in individual and foundation giving, and the need to develop and implement a strategy of fundraising from corporations should be evaluated and moved forward. It is of critical importance that the fundraising programs related to these revenue sources be built up and strengthened so as to provide BBRI with a solid and consistent Annual Fund program.

The revenue goals, however, should also be placed within the reality of the "marketplace", i.e. should be comparable to those of other basic research organizations somewhat similar to BBRI, e.g. Worcester Foundation or Jackson Labs. The fundraising revenue goals for BBRI should be realistic and therefore achievable, so as to create a sense of accomplishment and confidence among the solicitors and donors. One way of reaching this objective is to focus the Annual Fund on projects or programs, with clearly defined goals in each area. Following are suggestions for giving programs.

GIVING PROGRAMS

INDIVIDUALS

Board
Direct Mail
Special Events
Planned Giving
Major Gifts

The most significant addition to the current individual giving program is the development and implementation of a major gifts or special gifts program. Initially we will identify 5-10 major gift prospects and, with the assistance of volunteers, begin cultivating them for a major gift to BBRI. As we now have the Peabody Challenge in place, the major giving program will be focused on raising the $300,000 needed to meet the challenge and purchase the X-ray crystallography equipment.

CORPORATE

We are beginning to move forward with two facets of a corporate giving program: soliciting new corporate donors for BBRI through proposals (Boehringer Mannheim; Boston Scientific), and assessing the need for and development of a volunteer Corporate Committee.

FOUNDATIONS

We have, in the past, benefited from the generous support of local foundations. We should strive to maintain this support, while broadening our contacts to national foundations. The possibility of programmatic or individual support from national foundations such as the Howard Hughes Medical Institute, Kresge Foundation, Keck Foundation, etc., is underway and it is anticipated that several proposals will go to national foundations in the second half of the year. These foundations may be appropriate sources for major items of capital equipment to complement new programs or recruitments.

Looking forward to the long term Development focus, two needs of the Institute emerge that will require vigorous fundraising activity: increasing BBRI's endowment, and building new space for the Institute. As these cannot happen concurrently, strategic decisions need to be made as to the priority for future campaigns; however, it is essential that both are undertaken at some time in the future.

PUBLIC RELATIONS

Lack of communication with the general public appears to be a major impediment to soliciting charitable contributions to BBRI, and effort should be invested in improving the visibility of the work done at the Institute while making the importance of this work more understandable. Recognition of the longterm importance and value of investment in basic science requires considerable vision and sophistication on the part of BBRI supporters, and this challenge needs to be considered as the public relations activity moves forward. The focus of the Institute has been sharpened and the mission clarified without sacrificing the fundamental commitment to basic research; these objectives must be continued and strengthened. With the assistance of a public relations consultant, a solid, comprehensive foundation for increasing and improving BBRI's public relations efforts will be built and implemented. The three areas that the public relations activity will focus on strengthening and expanding are: 1: media relations; 2) communications/publications; and 3) government (federal, state, and local) liaison development.

Additionally, the visibility of the Institute can be raised by building bridges with the Harvard teaching hospitals and other area institutions, and by sponsoring high

quality symposia. With respect to <u>clinical interactions</u> , a work group is needed to outline the possibilities for interaction with clinical departments at Harvard hospitals. The combined expertise and interests could form the basis for a number of individual, high visibility collaborations as well as potential SCOR and Center grant applications. At the present time, opportunities appear to be greatest with regard to interactions with the Department of Medicine at the Beth Israel Hospital in Boston, where the new Director of the BBRI will continue to hold a Faculty position; however, possible collaborations with all Boston hospitals will be explored and considered.

Three specific initiatives will be considered: (1) An application for an NIH-funded training program to allow research-oriented clinical trainees at Boston-area hospitals to pursue 2-3 years of basic research training at the Institute. The cross-fertilization of expertise should be of interest to all parties involved; (2) the creation of a foundation (similar to Whitaker) to fund <u>interactive</u> projects between Boston-area hospitals and BBRI; members of both institution's governing boards will be invited to contribute, and peer review of grant applications will be performed by four individuals from the two institutions; (3) the Boston Collaborative Heart Failure Center.

In regards to <u>symposia</u>, an international symposium honoring the career of John Gergely will be held on July 27-29, 1996, and a second "Seidel Symposium" in the spring of 1997, continuing with a focus in the area of Muscle will be organized within the next two years and should become a regular event. Consideration should be given as to whether the focus should continue to be smooth muscle. Additionally, the idea of an event possibly co-sponsored by another research institute and the BBRI, possibly funded by a private foundation or covered by public broadcasting to investigate the future of basic biomedical research should be developed and, at the appropriate time, a work group should be formed to implement the project.

The BBRI public relations plan is composed of several strategies, each aimed at a very specific objective for the coming year. The foundation that should result from the development and implementation of these concurrent pieces will, hopefully, provide the base on which to continue to build in the future.

C. Investments

BBRI, through the collective efforts of its faculty and Development Office, has built up an investment portfolio of over $7,000,000. The portfolio is managed by the Investment Committee, comprised of Trustees and senior management staff of the Institute.

The Investment Committee has established a set of investment goals within defined parameters. Currently, the portfolio consists of mutual funds ranging from money market to aggressive growth funds. Historically, 5% of the average portfolio balance for the prior three years is available for annual operations. In addition, it is

anticipated that, over the long run, investment income will be sufficient to pace the principal growth with inflation.

D. Technology Transfer

Since 1982, BBRI scientists initiated a number of projects that were funded by corporate research contracts, with the three major contracts bringing $1.6 million in research funds to BBRI in the period 1982-1994. In addition, through the auspices of BBC, a solely-owned for-profit subsidiary of BBRI, Institute scientists have been awarded five phase I grants through NIH's Small Business Innovative Research (SBIR) program. Eight patents have been issued to BBRI, and one of these is just about to produce an income for BBRI from a licensing agreement.

No matter how impressive this record may be for an institute as small as BBRI, it was the result of sporadic initiatives by individual scientists and not the result of long-term planning. There is no question that a systematic technology transfer effort could have opened additional opportunities for corporate research and licensing agreements. In recognition of this important potential source of research funding and revenue, BBRI has recently become a member of the United Office of Technology Transfer of MBRI (Massachusetts Biotechnology Research Institute), which provides effective technology transfer services to a number of Boston area academic institutions.

In the past year, MBRI has conducted a detailed technology assessment of BBRI and has played an important educational role by increasing the awareness of BBRI scientists of technology transfer opportunities. This has set the stage for a systematic technology transfer effort, whose core will be the marketing of BBRI intellectual property to biotechnology companies, major pharmaceutical companies, and venture capital. An important component of this initiative will be to identify technology areas in which BBRI has particular strengths and market those as a package so as to enhance their impact and visibility. Another opportunity is offered by the SBIR funding mechanism, especially through the large phase II grants, which would require a reexamination of the role of BBC, including the possibility of joint ventures between BBC and established biotechnology companies. It is hoped that through aggressive efforts along these lines, research funding and licensing income from corporate sources will expand significantly above the current $100,000 annual average.

III. Expenses

A. Directs

The responsibility of budgeting for and monitoring the expenditure of direct costs associated with a specific grant has traditionally been held by the Principal Investigator who submitted the grant. Such costs include salaries and fringe benefits, supplies, publication and travel related to a grant. BBRI has always maintained a

policy of conserving research funds and has been very successful, thanks to the efforts of the faculty, in limiting unnecessary spending of grant-related revenue.

This system of allocating and monitoring grant-related revenue is in keeping with BBRI's overall philosophy of encouraging independence and creativity among the faculty, and we feel strongly that this policy is a valuable incentive in the recruiting process.

B. Indirects

The Institute has been, and continues to be committed to keeping its administrative staffing levels lean and multi-functioning. In many cases this means foregoing individuals hired specifically to perform functions such as purchasing, research administration and equipment maintenance and scheduling. Instead, systems are designed in such a manner to provide these functions with existing personnel, e.g. the Director acts as both Chief Executive Officer while managing a full time laboratory; the Assistant Director is responsible for all physical plant management as well as financial matters; Principal Investigators assume the purchasing role as well as pursuing and expanding their research projects.

The indirect cost rate will be negotiated in early 1997 and will take effect September 1, 1997. This is an area of critical concern to the Institute. There is a clear justification for an increase of the indirect cost rate. However, the Department of Health and Human Services is under pressure to lower indirect cost rates. We have made an assumption in the budget that our indirect cost rate will be increase by 10% of its current rate (to approximately 96% of personnel costs).

Although we have hit a particularly trying time for annual funding, we have a solid financial base, are debt-free, and have a commitment of financial and managerial support from the Board of Trustees. We believe the influx of new talent, combined with the scientific goals established in this plan, will be the foundation for our continued success.

BOSTON BIOMEDICAL RESEARCH INSTITUTE

POLICIES AND PROCEDURES MANUAL

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Last modified 8/28/03

FACULTY REAPPOINTMENT

Any Scientist, Principal Scientist, or Senior Scientist shall be notified one year before the term of his/her appointment expires whether the appointment will be recommended for renewal by the Committee on Research. Scientists who have received formal warnings that they may not be reappointed may be granted one-year extensions. The decision whether to give an extension will be made in a timely manner. If no formal warning is given, scientists are eligible for full term reappointments.

Amongst the criteria for considering reappointments are:

- Continued scientific productivity as evidenced by publications and grants.
- Service to the Institute.
- Scientific reputation.

Any Scientist, Principal Scientist, or Senior Scientist appointment not recommended for renewal shall be extended so as to provide one-half, one, or two years respectively, between the action and the expiration of the current appointment.

JOB DESCRIPTION

SCIENTIST

Scientists are appointed for three-year terms, Principal Scientists for four-year terms and Senior Scientists for five-year terms. These ranks correspond to the positions of Assistant Professor, Associate Professor, and Full Professor, respectively. Persons appointed to this category are classified as regular employees and may be on a full-time or part-time basis.

Qualifications:
The qualifications for an appointment are as follows:

Senior Scientist: established scientists of high standing internationally in their field as evidenced by at least two letters of recommendation by renowned colleagues.

Principal Scientist: should have repeatedly demonstrated the ability to compete successfully as a Principal Investigator for a research grant or equivalent productivity at the national level.

Scientist: usually has had at least three years of post-doctoral or equivalent experience prior to the appointment and is usually the Principal Investigator of a research project.

GRANTS
BRIDGE SUPPORT FOR FACULTY

FIRST YEAR

- A first year of bridge support in an amount equal to the PI's salary is given to productive members of the faculty contingent upon timely submission of competitive grant applications.

- The amount of bridge support is proportional to the percentage of the PI's time allocated to the specific grant whose funding is delayed.

- If funding is received before bridge support runs out, unused moneys will be returned to the Institute.

- Application does not require submission of pink sheets and scores but does require a financial statement.

- For faculty members who have previously received bridge support, a new round of bridge support can only be obtained after successfully obtaining a full R01-type grant (e.g. NIH, NSF, SBIR) of three years or longer, or comparable supports.

SECOND YEAR

- Only available in exceptional circumstances, provided the Board of Trustees approves the request.

- PI must submit to the COR with the application for a second year of support:
    - Scores and pink sheets from most recent NIH review
    - CV
    - Letter outlining the PI's plan for successfully building his/her program and obtaining funding

- Criteria to be used in making decision of whether to recommend second year of bridge support:
    - Probability of funding
    - Excellence of science
    - Contributions to Institute
    - Programmatic fit

BOSTON BIOMEDICAL RESEARCH INSTITUTE

BY-LAWS

[as amended through November 15, 2001]

separate affirmative vote of the Trustees and Faculty, taken one year prior to the expiration of the Director's term.

The Deputy Director shall be appointed annually by and from the Faculty for a one-year term, upon recommendation of the Director.

Section 3. Assistant Officers. The Board of Trustees may appoint an Assistant Treasurer, an Assistant Secretary-Clerk and such administrative officers as it may from time to time believe necessary. Such additional officers need not be members of the Corporation. They shall serve from the time of their appointment until the first meeting of the Board following the next succeeding Annual Meeting.

Section 4. Vacancies. Any vacancy occurring in the office of any of the officers of the Corporation, except that of the Director and the Deputy Director, may be filled at any time by the Board of Trustees, and any person elected to fill such a vacancy shall hold office for the remainder of the term of the Officer whose place he/she takes. In the event of a vacancy in the office of Director, the Deputy Director shall assume the office until such time as the Trustees appoint an acting Director. If the Deputy Director shall resign or be or become unable to serve for the remainder of the term, the Director, after consultation with the Committee on Research, may appoint an interim Deputy Director who shall assume the office for the remainder of the term.

Section 5. Number of Offices Held. Except as otherwise specifically required by law or as otherwise specifically provided by these By-laws any person may hold two or more offices in the Corporation at the same time.

ARTICLE V

FACULTY

Section 1. Faculty. All Staff Scientists holding appointments for terms of not less than three (3) years shall constitute the Faculty. It shall from time to time advise the Committee on Research on matters relating to the scientific and educational activities of the Corporation, except as otherwise provided in these By-laws.

Section 2. Tenure.

A. The Board of Trustees may from time to time grant an appointment with tenure to a staff scientist at the Institute. Such appointments shall be made only on the recommendation of an ad hoc tenure committee to be appointed by the Board of Trustees, and with the approval of two-thirds of the members of the Committee on Research.

B. **Tenure, as such term is used in the By-laws, shall mean a permanent appointment to the staff of the Institute and a guarantee of the reasonable use of the research laboratories or other facilities of the Institute consistent with the research funding available to the staff member** subject to the following conditions:

1. Tenure shall terminate on retirement or upon resignation of the staff scientist or pursuant to revocation for grave misconduct or neglect of duty by a vote of two-thirds of the members of the Board of Trustees, upon the recommendation of the Committee on Research.

2. Tenure may be withdrawn by the Board of Trustees upon recommendation of two-thirds of the members of the Committee on Research if the research being conducted by that individual becomes inconsistent with the research program of the Institute.

3. Such further conditions as may be imposed by the Committee on Research in individual cases.

4. All determinations by the Board of Trustees hereunder shall be final and binding on all persons.

ARTICLE VI

DUTIES OF OFFICERS

Section 1. Chairman. The Chairman shall be the senior officer of the Corporation. He/she shall cause to be prepared the agenda of the Annual Meeting and of all special meetings of the Corporation in cooperation with the Director, and he/she shall preside at all meetings of the Corporation. In addition, he/she shall perform such other special duties as from time to time may be determined by the members of the Corporation.

Section 2. President. The President shall preside at the meetings of the Board of Trustees, and he/she shall cause to be prepared the agenda of the meetings of said Board in cooperation with the Director.

Section 3. Vice Presidents. The Vice Presidents shall assist the President in the performance of his/her duties and shall act for the President in the latter's absence.

Section 4. Director. The Director shall be the chief executive officer of the Corporation. He/she shall report to and carry out the general policies established by the Board of Trustees, which shall regularly review the performance of the Director. As chief executive officer he/she shall be responsible for the direction, management and implementation of the scientific and educational activities and policies of the Corporation consonant with the determinations of the Committee on Research with respect to all matters related to such activities. In addition, he/she shall perform such other duties and have such other powers as the Board of Trustees may from time to time determine. The Director shall preside at the meetings of the Committee on Research.

Section 5. Deputy Director. The Deputy Director shall assist the Director in the performance of his/her duties and in his/her absence shall act for the Director.

Rating: Moody's: "Baa3"
Standard & Poor's: "BBB-"
(See "Ratings" herein)

NEW ISSUE

In the opinion of Ropes & Gray, Bond Counsel to the Institution, under existing law, interest on the Series 1999 Bonds is excluded from the gross income of the owners of the Series 1999 Bonds for federal income tax purposes, assuming continued compliance by the Issuer and the Institution with the Internal Revenue Code of 1986, as amended. Interest on the Series 1999 Bonds is not an item of tax preference for purposes of the federal alternative minimum tax imposed on individuals and corporations. However, interest on the Series 1999 Bonds will be taken into account in determining adjusted current earnings for the purpose of computing the alternative minimum tax imposed on certain corporations (as defined for federal tax purposes). In the opinion of Bond Counsel to the Institution, under existing law, the Series 1999 Bonds and any income derived therefrom, including any income from any sale, exchange or transfer of the Series 1999 Bonds, shall at all times be free from Massachusetts taxation, although the Series 1999 Bonds and the interest thereon are included in the measure of Massachusetts estate and inheritance taxes and of applicable Massachusetts corporation excise and franchise taxes. For federal and Massachusetts tax purposes, interest includes original issue discount. See "TAX EXEMPTION" herein.

[redacted]

$17,000,000
MASSACHUSETTS DEVELOPMENT FINANCE AGENCY
Revenue Bonds
(Boston Biomedical Research Institute, Inc. Issue - Series 1999)

Dated: February 1, 1999 **Due: February 1, as shown below**

The Massachusetts Development Finance Agency Revenue Bonds (Boston Biomedical Research Institute, Inc. Issue - Series 1999) (the "Series 1999 Bonds") are issuable only as fully registered bonds without coupons and, when issued, will be registered in the name of Cede & Co., as Bondowner and nominee for The Depository Trust Company ("DTC"), New York, New York. DTC or its custodial agent will act as securities depository for the Series 1999 Bonds. Purchases of the Series 1999 Bonds will be made in book-entry form, in the denomination of $5,000 each or any integral multiple thereof. Purchasers will not receive certificates representing their interest in Series 1999 Bonds purchased. So long as Cede & Co. is the Bondowner, as nominee of DTC, references herein to the Bondowners or registered owners shall mean Cede & Co., and shall not mean the Beneficial Owners (as hereinafter defined) of the Series 1999 Bonds.

Principal and semiannual interest on the Series 1999 Bonds will be paid by State Street Bank and Trust Company, as trustee (the "Trustee") and paying agent. So long as DTC or its nominee, Cede & Co., is the Bondowner, such payment will be made directly to DTC. Disbursement of such payments to the DTC Participants is the responsibility of DTC and disbursements of such payments to the Beneficial Owners is the responsibility of the DTC Participants, all as more fully described herein. Interest will be payable on August 1, 1999 and semiannually thereafter on February 1 and August 1 to the Bondowners of record as of the close of business on the fifteenth day of the month preceding such interest payment date.

The Series 1999 Bonds shall be special obligations of the Massachusetts Development Finance Agency (the "Issuer") doing business as "Mass Development".

MASSDEVELOPMENT

The Series 1999 Bonds will be issued pursuant to a Loan and Trust Agreement dated as of February 1, 1999 (the "Agreement") among the Issuer, Boston Biomedical Research Institute, Inc., (the "Institution") and the Trustee. The Issuer will loan the proceeds of the Series 1999 Bonds to the Institution. The Series 1999 Bonds are payable solely from and secured by payments to be made to the Trustee for the account of the Issuer by the Institution and from such other funds as may be available therefor under the Agreement. Reference is hereby made to this Official Statement for pertinent security provisions of the Series 1999 Bonds.

THE SERIES 1999 BONDS DO NOT CONSTITUTE A GENERAL OBLIGATION OF THE ISSUER OR A DEBT OR PLEDGE OF THE FAITH AND CREDIT OF THE ISSUER OR A DEBT OR PLEDGE OF THE FAITH AND CREDIT OF THE COMMONWEALTH OF MASSACHUSETTS OR ANY POLITICAL SUBDIVISION THEREOF; EXCEPT TO THE EXTENT PAID FROM BOND PROCEEDS, THE PRINCIPAL OF, PREMIUM, IF ANY, AND INTEREST ON THE SERIES 1999 BONDS ARE PAYABLE SOLELY FROM THE REVENUES AND FUNDS PLEDGED FOR THEIR PAYMENT IN ACCORDANCE WITH THE AGREEMENT. THE ISSUER HAS NO TAXING POWER.

MATURITIES, AMOUNTS, RATES, AND PRICES OR YIELDS

Due February 1	Principal Amount	Interest Rate	Price or Yield	Due February 1	Principal Amount	Interest Rate	Price or Yield
2001	$255,000	5.00%	4.25%	2006	$330,000	5.00%	4.85%
2002	270,000	5.00	4.45	2007	345,000	5.00	4.95
2003	285,000	5.00	4.60	2008	360,000	5.00	100
2004	295,000	5.00	4.70	2009	380,000	5.10	100
2005	310,000	5.00	4.75				

$5,170,000 5.65% Term Bonds Due February 1, 2019 to yield 5.68%
$9,000,000 5.75% Term Bonds Due February 1, 2029 - Price 100%
(Accrued interest to be added from February 1, 1999)

THE SERIES 1999 BONDS ARE SUBJECT TO REDEMPTION PRIOR TO MATURITY AT VARIOUS PREMIUMS, OR AT PAR UNDER CERTAIN CIRCUMSTANCES, AS MORE FULLY SET FORTH HEREIN.

The Series 1999 Bonds will be offered, when, as and if issued and accepted by the Underwriter, subject to prior sale, to withdrawal or modification of the offer without notice, and opinions as to legality and certain other matters by Ropes & Gray, Boston, Massachusetts, Bond Counsel to the Institution. Certain legal matters will be passed upon for the Institution by its counsel, Ropes & Gray, Boston, Massachusetts. Certain legal matters will be passed upon for the Underwriter by its counsel, Edwards & Angell, LLP, Boston, Massachusetts. The Series 1999 Bonds are expected to be available for delivery to DTC in New York, New York, or its custodial agent, on or about February 19, 1999.

State Street Bank and Trust Company

February 11, 1999

The October 1995 revision to OMB Circular A-122 went into effect on September 29, 1995. This revision allows interest expense to be recoverable from federal sources. Before the passage of this revision, most research institutes leased facilities rather than financed the purchase of the facility with debt because only lease expenses were subject to indirect cost recovery. Due to the change in these interest recovery regulations, interest is now an allowable cost recovery item on capital expansion. Management has alerted the Department of Human and Health Services as to its plans to issue debt to fund the new laboratory facility and expects to pass on a significant portion of the new debt service when the Institute's new negotiated direct cost recovery rate goes into effect on July 1, 1999.

Balance Sheet. Management has placed an ongoing emphasis on liquidity and cash flow. The following table depicts the Institute's unrestricted cash and investments, operating expenses (less depreciation and amortization), and days cash on hand for the last five fiscal years. Days cash on hand is calculated by multiplying the Institute's unrestricted cash and investments by 365 (days/year) and dividing the results by total operating expenses less depreciation and amortization expenses.

Unrestricted Cash and Investments
(Market Value)

	1994	1995	1996	1997*	1998
Unrestricted Cash and Investments	$6,596,153	$7,503,434	$7,086,419	$7,701,747	$8,376,795
Operating Expenses					
(less depreciation & amortization)	$6,479,642	$6,545,906	$6,529,901	$4,993,143	$6,608,281
Days Cash on Hand	371.6	418.4	396.1	469.2	462.7

** Ten months of operating expenses calculated from August 31 to June 30 (303 days)*

The Institute's unrestricted cash and investments increased from $6,596,153 in 1994 to $8,376,795 in 1998, an increase of 27%. In 1996, days cash on hand decreased from 418.4 to 396.1 due to a $780,000 decrease in cash reserves. This is primarily due to the Institute's recruiting strategy which required the Institute to support two new investigators until they received grant funding. As of June 30, 1998, less than $500,000 of the Institute's $8.8 million of investments are permanently restricted.

Similarly, total net assets of the Institute has grown significantly, increasing from $8 million in 1994 to approximately $11 million as of June 30, 1998. Management attributes this growth to investment appreciation and a growing fundraising program.

Budget Procedures and Financial Controls

The Chief Operating Financial Officer meets with the Institute's scientists to draft a budget proposal for the following fiscal year. The Budget and Finance Committee reviews the budget in detail before submitting it for approval by the full Board. The Board sets the directives for the budget. At the June Board meeting, the final proposal for the following year is submitted for Board approval. Management reviews budget and expenditures each month and reports any variance to the Budget and Finance Committee.

Investments

As of January 15, 1999 BBRI had total investments of $8,495,289, at market value, of which $7,696,666 is unrestricted, $335,574 is temporarily restricted and the remainder is restricted. Currently, the investment portfolio is invested in mutual funds with an allocation of approximately 80% equities and 20% fixed income.

BOSTON BIOMEDICAL RESEARCH INSTITUTE

COMMITTEE ON RESEARCH

November 27, 1996

A regular meeting of the Committee on Research was held on Wednesday, November ~~28~~ 27, 1996, at 10:00 a.m. Present at the meeting were Drs. Morgan, Grabarek, Paulus, Raso, and Wang. Others present were Mr. Thomas McQuaid, and Ms. Barbara Zillman, who recorded the proceedings.

1. Dr. Morgan asked for approval of the minutes of October 29, 1996. Clarification was requested on three items. They were as follows:

1) In item 5, concerning Dr. Volloch, the term "bridge support" is inaccurate and should be redefined as "R21 support." 2) Same sentence, the term "scientific misconduct" is inaccurate and should be restated as "alleged misconduct...." 3) In item 6, concerning potential BBRI floor space, the discussion is more accurately reflected with the following addition after the phrase ".....room itself belongs to BBRI.": "Dr. Grabarek asked if the room is available immediately and Mr. McQuaid replied that it is not."

With these clarifications noted, it was

VOTED: Unanimously, to approve the minutes of October 29, 1996.

Following this vote, Dr. Morgan recapped for the record, because of subsequent events, the intent of the C.O.R., at its last meeting, to provide Dr. Volloch with temporary laboratory space following his relocation from the second floor. Dr. Leavis had volunteered to clear one and one-half of his own benches for use by Dr. Volloch, with the understanding that if Dr. Leavis was awarded his then-pending grant application, his space would be returned. Dr. Leavis has since learned that his grant application has been awarded and he will need to reclaim his lab for expansion of his program. The ESR room in the basement was made available, temporarily, to Dr. Volloch by Dr. Graceffa. Dr. Graceffa and Dr. Gergely are composing a letter to Dr. Volloch stating that he is to vacate the ESR room by a specified date and that while occupying it, there are not to be radioactives used in that room, and that the equipment must not be harmed. C.O.R. members agreed with the appropriateness of giving Dr. Volloch such a letter. The following issues were reiterated: the allocation of space in the basement to Dr. Volloch is temporary; the C.O.R. as a body, and each member individually, must work to see that Dr. Volloch submits a follow-up competitive renewal grant application to the NIH - the conditions of the R21 that Dr. Volloch received require this and assure expedited review if he submits his application on time; the C.O.R. must consider upon submission of that grant application, what to do next. The decision concerning his application should be known in July. If triaged, Dr. Volloch will be asked to leave the Institute but if funded, he may be allowed to stay. Funding must be from the NIH; an Alzheimer's grant would not be enough. If asked to leave, he is to be given advance notice (if outcome is in July, notification would be for relocation in September). In any case, he has been made no promise as to continued lab or office space beyond his current funding. If during his accelerated review, his score is in the top 30%, he will be allowed to resubmit his application for the summer deadline.

2. The next item of discussion was setting a policy on misconduct proceedings. There was some discussion about this topic and it was agreed to continue without formal policy implementation.

3. Proposals were discussed next. The first was that of Dr. Coleman.

Dr. Coleman has made a request for his first year of bridge support (see attached). Using the current guidelines for granting requests for bridge support, Dr. Coleman was evaluated on several items and concerns were raised by C.O.R. members. Dr. Grabarek noted that Dr. Coleman has not been active in publishing since 1994. Dr. Paulus noted that Dr. Coleman had been appointed to a faculty position at the Institute with two active NIH grants, and never re-applied for the grants when they

expired. Further, he has made himself very part-time at the Institute. A formal vote by the C.O.R. in October, 1991 guaranteed him a salary for three years, contingent upon his funding. Dr. Coleman now has no funding. C.O.R. members concluded that by current guidelines as well as the vote of the C.O.R. in October, 1991, Dr. Coleman does not qualify for full bridge support. Some further discussion took place and it was moved, and by a majority,

VOTED: Because of questions of productivity and failure to renew grant applications in a timely fashion, but because of past contributions to the Institute, three months salary support will be awarded to Dr. Coleman, using his base salary of $70,000. This is consistent with the determination of the October 2, 1991 meeting of the C.O.R.

There were four aye votes and one ~~nay vote~~. abstention by Dr Vaso.

The second proposal to be discussed was that of Dr. Raso. He left the room while discussions took place.

Dr. Raso is requesting his second year of bridge support (see attached). His first year of support was awarded before the current criteria were established and his first year of support was very generous. The merits of Dr. Raso's proposal were deemed to be that he had brought in over a $1,000,000 in overhead to the Institute. Furthermore, Dr. Raso has viable patents and is very technology-oriented, a future direction the Institute will take, and a probable revenue stream. The negative aspects of the proposal were considered to be a lack of publications. The amount to be requested was further refined by again referring to current criteria. Dr. Raso has already received an equivalent of 16 months of support, including supplies, in his first year of support. Therefore, a financial commitment of eight months will be made to him. It was,

VOTED: Unanimously, to request a second year of bridge support for Dr. Raso from the Board in an amount equivalent to eight months of both salary and supplies (at $10,000 per year), and to allow that amount to be awarded effective immediately. This vote is subject to ~~reversal~~ approval by the Board at its next meeting in January, 1997.

4. There being no Other Business, the meeting was adjourned at 1:35 p.m.

Barbara Zillman

Barbara Zillman

December 9, 1996

BOSTON BIOMEDICAL RESEARCH INSTITUTE

Victor A. Raso, Ph.D.

20 STANIFORD STREET, BOSTON, MASSACHUSETTS 02114
Area code 617 • 742-2010
Telefax 617 • 523-6649

November 21, 1996

To The Members of The Committee On Research:

At the end of November funds will run out on a 6-month Small Business Innovation Research Grant "Catalytic Antibodies for the Treatment of AIDS." This money (99,911 - 38,174 BBRI service fee) has been sustaining my laboratory following the finish of institutional support. Unfortunately, it now appears that the re-submitted competing continuation of my NIH grant "Targeting Toxins with Acid-Triggered Hybrid Antibodies" will not be funded this round. I am therefore writing to request institutional funds for second year support to temporarily operate the laboratory (one year salary plus supplies) while NIH and other funding is being sought.

The prospects for future support are as follows:

1. Experiments are underway to obtain data for second phase funding of the recently awarded 6-month Small Business Innovation Research Grant "Catalytic Antibodies for the Treatment of AIDS".

2. A similar NIH R01 grant "Catalytic Antibodies Directed Against HIV" will also be submitted.

3. My NIH grant "Targeting Toxins with Acid-Triggered Hybrid Antibodies" will either be re-submitted for competing renewal or more-likely will be revised as a new grant "Targeting Binary Toxins" to better reflect its renovated scientific direction.

4. A new NIH grant "Amyloid ß-Peptide Equilibria in Alzheimer's Mice" is currently being written.

5. A related grant, "Catalytic Antibodies for the Treatment of Alzheimer's Disease" will be submitted as an SBIR grant and also to the Alzheimer's Association.

Given the scope and relevance of the topics addressed, I believe that at least one of these grants will be funded.

To help strengthen the competing renewal grant application, I have submitted two manuscripts that are currently being reviewed by the *Journal of Biological Chemistry*.

I believe that I have made a positive contribution to the BBRI over the years and with eventual funding hope to do so in the future. While the "fit" of my research may not be ideal in the current scheme of things, it has always been on the cutting edge of biomedical research and has excited interest among our non-scientist supporters.

With regard to personnel, Christine Kearney is currently providing essential technical assistance on the AIDS, cancer therapy and Alzheimer's disease projects in addition to maintaining the smooth operation of all laboratory support functions. I will utilize my salary to keep Christine working at the BBRI.

Sincerely,

Vic Raso

Vic Raso, Ph.D.

November 21, 1996

BRIDGE SUPPORT

FIRST YEAR

- A first year of bridge support in an amount equal to the PI's salary is given to productive members of the faculty contingent upon timely submission of competitive grant applications.
- The amount of bridge support is proportional to the percentage of the PI's time allocated to the specific grant whose funding is delayed.
- If funding is received before bridge support runs out, unused moneys will be returned to the Institute.
- New faculty are required to have used all of their seed money before being eligible to request bridge funding and to have successfully brought in a grant submitted from the BBRI in order to be eligible.
- Application does not require submission of pink sheets and scores but does require a financial statement.
- For faculty members who have previously received bridge support, a new round of bridge support can only be obtained after successfully obtaining a full RO1-type grant (e.g. NIH, NSF, SBIR) of three years or longer, or comparable support.

SECOND YEAR

- Only available in exceptional circumstances until additional sources of revenue are available to the Institute.
- PI must submit to the COR with the application for a second year of support:
    - Scores and pink sheets from most recent NIH review
    - CV
    - Letter outlining the PI's plan for successfully building his/her program and obtaining funding
- Criteria to be used in making decision of whether to recommend second year of bridge support:
    - Probability of funding
    - Excellence of science
    - Contributions to Institute
    - Programmatic fit

Begin to write your grant proposal EARLY.

The 10/88 revision of the PHS-398 Instructions states, "PHS estimates that it will take from 10 to 15 hours to complete this application. This includes time for reviewing the instructions, gathering needed information, and completing and reviewing the form." The 9/91 revision states, "The PHS estimates that it will take approximately 50 hours to complete this application for a regular research project grant. This estimate does not include time for development of the scientific plan. Items such as human subjects are cleared and accounted for separately, and are therefore also not part of the time estimate for completing this form." I have heard occasional stories of proposals that were written a week before the deadline and were funded. However, in my experience and that of many of my colleagues it usually takes an appreciably longer time—several weeks to several months—to prepare a good grant application. And this is not counting the many months that may be required to accumulate the preliminary data on which the proposal is based! Moreover, it is important also to set aside time to have others read the proposal (about 1 month), for you to consider—and act on—their relevant suggestions (about 1 month), and for the grants office at your institution to check over the administrative aspects of the proposal (generally between 5 days and 2 weeks). A hastily prepared application is often a poorly prepared application.

Think about the time required to do each of the following:

- Compile the relevant data.
- Prepare tables, figures, and/or photographs.
- Finish the various stages of the project: outline, first draft, second draft, etc. Set deadlines for yourself for finishing each stage.
- Have others read your proposal. Have a good second draft of the proposal ready to send to colleagues for appraisal at least 9–10 weeks before the grant application

OIPE IAP82 JUL 2 8 2007 PATENT & TRADEMARK OFFICE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):	Victor Raso
Application No.:	09/992,994
Filing Date:	November 6, 2001
Title:	IMMUNOLOGICAL CONTROL OF β-AMYLOID LEVELS *IN VIVO*
Art Unit:	1652
Examiner:	Patterson, C.

DECLARATION OF KEVIN M. FARRELL, ESQ.

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Kevin Farrell, do hereby declare and say:

1. I am a Partner at the law firm of Pierce Atwood focusing on the preparation and prosecution of biotech patent applications. I joined Pierce Atwood in 2002 from my own firm, Farrell & Associates, P.C. I received a B.S. in Biology from Boston College (1982) and a J.D. from Northeastern University School of Law (1989).

2. As the Founding Principal of Farrell & Associates, I was intimately familiar with the firm's business practices and record keeping. In the ordinary course of business, timekeepers working on a patent application would read and prepare documents, including notes they made, and those documents and notes would be kept in the file for

that patent application. In addition, timekeepers recorded their time. The bills for each application were also saved in the ordinary course of business.

3. When I joined Pierce Atwood, I retained ~~all the~~ business records of Farrell & Associates. The Exhibits I discuss below are from those business records. KMF 3/29/07

4. Shortly before January 27, 1999, I spoke to Ms. Pamela Torpey, a patent agent representing the Boston Biomedical Research Institute (BBRI) as an outside consultant. Ms. Torpey asked if we could prepare a patent application relating to the work of Victor Raso, Ph.D. regarding an Alzheimer's vaccine. We discussed the invention briefly, and she sent the disclosure to me at Farrell & Associates on January 27, 1999 [Ex. A].

5. We received the referenced correspondence from Ms. Torpey, with enclosures, on February 1, 1999 [Ex. A]. I acknowledged receipt of the disclosure [Ex. B] and studied the disclosure that same day [Ex. C].

6. For efficiency reasons, I assigned the application to a full-time Technical Specialist working for me, Shayne Y. Huff, Ph.D. Shayne and I spoke about the disclosure, and she studied it on February 5, 1999 [Ex. C] and prepared a memorandum to the file regarding her analysis of the disclosure on February 8, 1999 [Ex. D; Ex. C].

7. Shayne spoke with Dr. Raso on February 23, 1999 concerning patentability [Ex. E; Ex. C] and prepared a memorandum summarizing that conversation [Ex. F].

8. We received a facsimile from Dr. Raso on February 26, 1999 with further information in response to his conversation with Shayne [Ex. G]. I studied those materials that day and spoke to Shayne regarding them [Ex. H]. Shayne studied them on March 1, 1999, began drafting claims, and spoke to me about the claims that day [Ex. H].

9. On March 2, 1999, the next day, Shayne spoke with Dr. Raso with questions concerning the invention, sent him a fax requesting further information, and continued drafting claims [Ex. I; Ex. H]. Shayne continued studying the disclosure materials and drafting claims and examples for the application on March 2 and 4, 1999 [Ex. H].

10. Dr. Raso sent us further data on March 5, 1999 [Ex. J], which Shayne incorporated into the examples on March 8, and 9, 1999 [Ex. H].

11. Shayne revised the draft claims and considered some potential prior art she had ordered on March 10, 1999 [Ex. H]. I reviewed the draft claims and examples that day and spoke to Shayne about them [Ex. H].

12. Shayne sent the draft claims to Dr. Raso and reviewed his comments on March 17, 1999 [Ex. H]. She also requested further information from Dr. Raso that day [Ex. H].

13. The next day, March 18, 1999, Shayne revised the examples, sent them to ~~Vic~~ Dr. Raso, vmf 3/29/07 and began drafting the Detailed Description section of the application [Ex. H]. Shayne continued to draft and revise the Detailed Description, as well as the Background of the Invention on March 19, 22, and 23, 1999 [Ex. H]. On March 24, 1999, she spoke with Dr. Raso regarding these sections, and she revised them on March 25, 1999 in response to further materials transmitted by the inventor [Ex. H].

14. Shayne further revised the Detailed Description section on March 29, 1999, and requested further information from Dr. Raso on March 31, 1999 [Ex. H]. On April 15, 19, and 22, 1999, Shayne contacted Dr. Raso requesting further information [Ex. K].

15. Shayne studied the comments she received from ~~Vic~~ Dr. Raso in response to her various vmf 3/29/07 requests on May 4, 1999 and revised the application accordingly [Ex. L]. She also received further comments from Dr. Raso and incorporated them into the application on

May 12, 1999 [Ex. L]. I studied the draft application and sent it to Ms. Torpey for review on May 13, 1999 [Ex. M].

16. Dr. Raso and Shayne spoke about his comments on the draft application on May 20, 1999, and Shayne revised the application accordingly [Ex. L]. She further revised the application on May 21, 1999 and May 25, 1999 and sent it to Dr. Raso and Ms. Torpey [Ex. L; Ex. N].

17. Shayne revised the application to include Dr. Raso's final comments [Ex. N], spoke to Dr. Raso, and finalized the application on June 14, 1999 [Ex. O]. We filed the final application, with its accompanying papers, on June 16, 1999.

18. As the Principal of Farrell & Associates, I was quite familiar with Shayne's workload and docket. As I recall, and as evidenced by the Exhibits discussed above, she took up the subject application in its chronological order. She did not give preference to other work unless the other work came earlier than Dr. Raso's in chronological order, or unless the other work was subject to deadlines established by the Patent Office (such as responses to Office Actions) or statutory bars.

I understand that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified patent.

Kevin M. Farrell, Esq.

Torpey Associates
P.O. Box 981 Douglas, MA 01516 tel. (508) 476-0002 fax (598) 476-9174

January 27, 1999

RECEIVED

FEB 0 1 1999

KEVIN M. FARRELL, P.C.
MAINE

Kevin Farrell, Esq.
Farrell & Associates, P.C.
12 Riverwood Dr.
P.O. Box 999
York Harbor, ME 03911

Dear Kevin,

It was a pleasure speaking with you the other day. Enclosed please find the information for the Raso invention. I would appreciate a copy of any draft applications for review. This will be a provisional application. Please do not hesitate to call if you have any questions.

On another matter, I have also enclosed my resume. If you know or hear of any opportunities I may pursue please let me know. I will be taking the Patent Bar in April. I look forward to hearing from you.

Sincerely,

Pamela Torpey

Pamela Torpey

Invention Disclosure Form

1. What is the technical field? Please be specific.

2. What problem are you trying to solve? Give references.

3. How have others tried to solve this problem in the past?

4. How has the prior solution failed to completely solve this problem?

5. Describe your solution concisely, including a summary of the results achieved, futher experimental work planned and any additional information that would be helpful

6. Depict your solution using at least one drawing.

7. Referring to the drawing, describe how your solution works. Describe with enough detail that another scientist will be able to duplicate the work

8. Describe the functional and/or structural differences between your solution and the prior solution:

9. Why do you believe your solution would not have been obvious to another inventor working on the same problem at the same time

10. Describe all results achieved by your solution:

11. Describe the advantages of you solution over the prior solution

Raso, Victor

Invention Disclosure -- Immunological Approaches to Controlling Beta-Amyloid Levels in the Body

1) This invention makes use of immunological technology as it applies to methods and reagents for controlling the levels of beta-amyloid which exist in the body. The invention utilizes the principles of immunology to design and develop specific anti-beta-amyloid antibody reagents and/or beta-amyloid vaccines to achieve this end.

2) Presently there is no way to modulate the concentration of beta-amyloid either in the peripheral circulation or in the brain where this peptide can precipitate to form cerebral plaques. The invention would provide a safe and highly specific method to effectively reduce the levels of this peptide in the body. An ability to modulate beta-amyloid levels could be important since there are numerous lines of evidence which indicate that the beta-amyloid peptide is either the direct or indirect cause of Alzheimer's disease (1-8).

3) As far as I know no one has previously attempted to control the levels of beta-amyloid which exist in the body.

4) See above.

5) My solution uses specific anti-beta-amyloid antibodies as a means to safely control the level of this peptide in either the peripheral circulation or in the brain. Once introduced into the body, these antibodies can effectively bind all of the soluble beta-amyloid which is present. Less beta-amyloid would then be available to form insoluble deposits which can cause disease lesions such as the cerebral plaques seen in Alzheimer's disease.

Endogenous systemic anti-beta-amyloid antibodies have been elicited by active immunization of animals using any of the beta-amyloid peptide antigens (vaccines) that have been synthesized in my laboratory as well as in other laboratories. Alternatively selected anti-beta-amyloid monoclonal antibodies which have been produced and isolated in my laboratory and others can be used as a form of passive immunization by directly infusing them into the blood stream or brain.

A variation on this theme includes the use of catalytic antibodies (9-12) that can selectively and repetitively destroy beta-amyloid molecules in the body. Likewise, antibodies which can dissolve preestablished beta-amyloid plaques have been described (13, 14) and could be used to diminish those harmful deposits.

Antibodies in the circulatory system will bind beta-amyloid in the blood stream and surrounding tissues but normally can not enter the brain. Intracerebral infusion of these anti-beta-amyloid antibodies is one feasible way to deliver them directly into the brain. However an easier approach for sequestering cerebral beta-amyloid has been devised. Delivery of systemic anti-beta-amyloid antibodies across the blood-brain barrier and into the brain can be be achieved by creating vectorized (15-18), bispecific reagents. These react with receptors on the cerebral capillaries and are transported into the brain by transcytosis. Once inside the brain anti-beta-amyloid antibodies can interact with either the soluble peptide or insoluble peptide aggregates and thereby lower the effective concentration of cerebral beta-amyloid. Reduced levels of beta-amyloid in the brain would diminish the chances of forming new cerebral plaques and could even dissolve preestablished plaques.

6) The following drawings help to depict my solution:

$$A\beta_n(\text{agg.}) \leftrightarrow nA\beta$$

$$A\beta_n(\text{agg.}) \rightarrow nA\beta + Ab \rightarrow nA\beta{:}Ab$$

Fig. 1 Effect of Anti-beta-amyloid Antibody (Ab) on Soluble Beta-amyloid (nAß) Versus Aggregated Beta-amyloid (Aß$_n$) Equilibrium

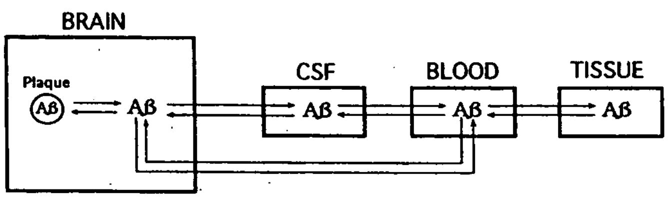

Fig. 2 Possible Equilibria of Beta-amyloid (Aß) Pools in the Body

CSF = Cerebro-spinal fluid

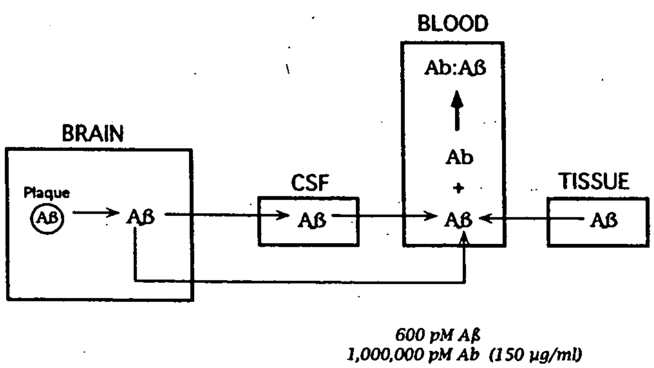

Fig. 3 Beta-amyloid (Aß) Equilibrium Altered by Specific Anti-Aß Antibody

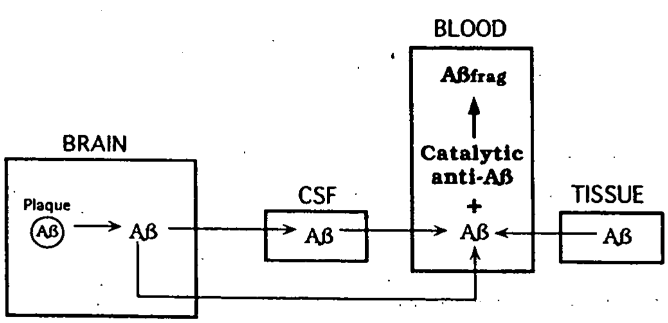

Fig. 4 Beta-amyloid (Aß) Equilibrium Altered by Catalytic Anti-Aß Antibody (Ab)

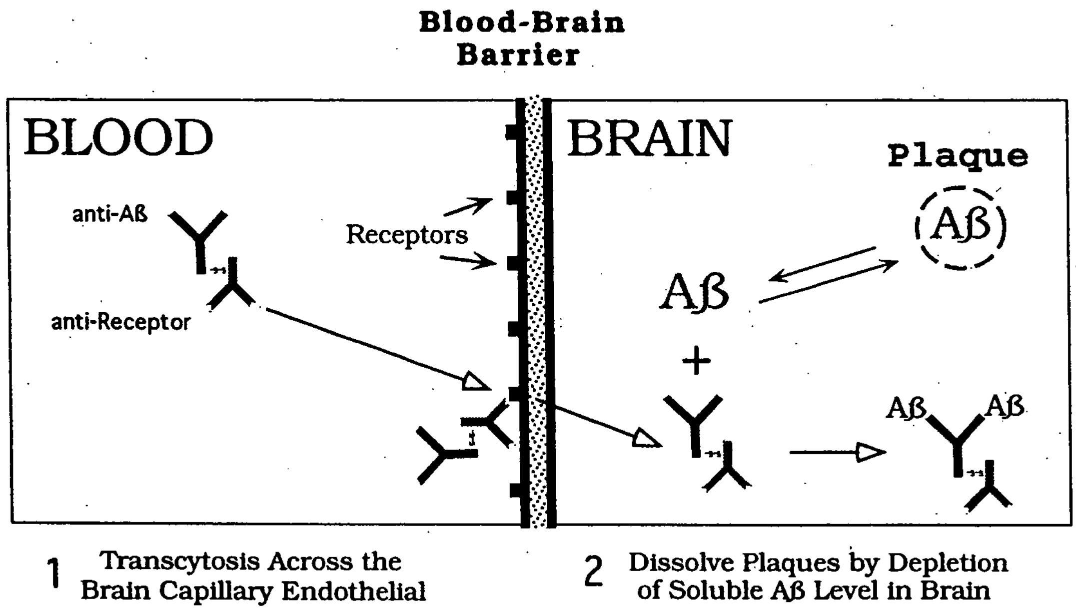

Fig. 5 Vector-Mediated Delivery of Anti-Aß into the Brain

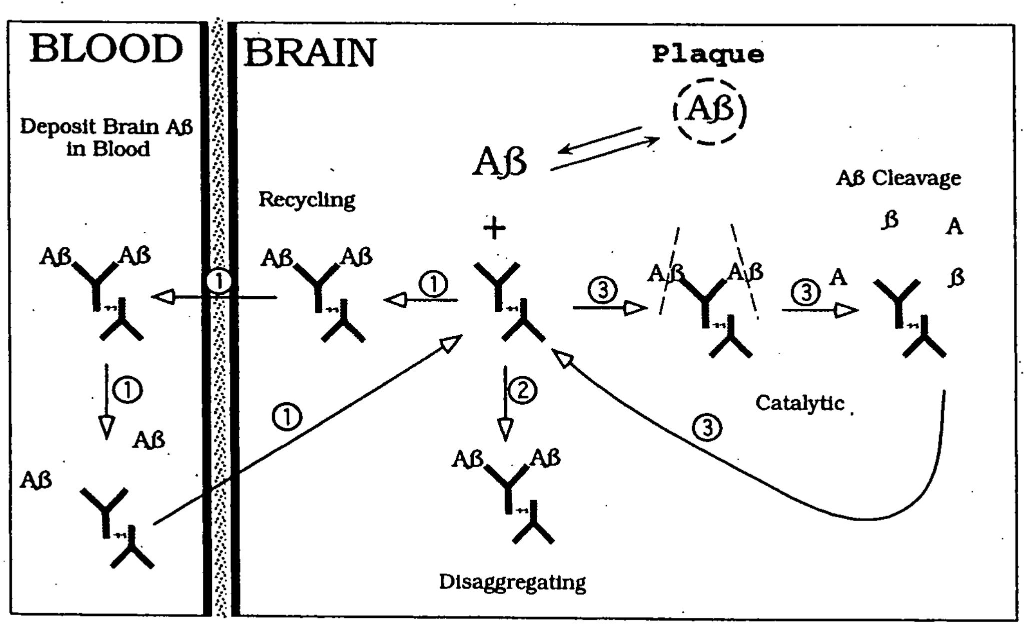

Fig. 6 Three Modes of Anti-Aß-Mediated Depletion of Beta-amyloid (Aß) from the Brain

7) Several important radioisotopic exchange experiments (19, 20) suggest that a dynamic equilibrium exists between soluble beta-amyloid (nAß) and fibrillar beta-amyloid aggregates ($Aß_n$) deposited as plaques in the brain (Fig. 1). Moreover, it has been demonstrated that treatment with certain anti-beta-amyloid monoclonal antibodies (Fig. 1) can effectively dissolve preformed Aß aggregates (13). Since these antibodies also inhibit the initial aggregation of the peptide (14), the results indicate that self-aggregates of beta-amyloid ($Aß_n$) are in equilibrium with a small amount of the beta-amyloid monomer (nAß).

Free beta-amyloid present in the blood most likely arises from peptide released by proteolytic cleavage of beta-amyloid precursor protein present on cells in the peripheral tissues (Fig. 2). Likewise most of the different free beta-amyloid species found in the brain and cerebrospinal fluid are probably derived from peptide released by secretase cleavage of beta-amyloid precursor protein expressed on brain cells. However intercommunication between these pools is a distinct possibility (Fig. 2) since the peptides are identical regardless of origin. If soluble beta-amyloid is the precursor of amyloid plaques and these pools do mix then senile plaques may be composed of both beta-amyloid originating in the brain as well as beta-amyloid that has been transported into the brain from the periphery. The possibility that blood-borne beta-amyloid might contribute to senile plaque formation in Alzheimer's disease is supported by the presence of capillaries at the core of plaques in the brain (21), deposits of beta-amyloid in cerebral blood vessels (22, 23) and immunocytochemical indications of beta-amyloid diffusing out of cerebral capillaries into the surrounding parenchyma (24). Radioisotopic studies indicate that soluble beta-amyloid in the blood can rapidly enter the brain and cerebrospinal fluid by passage across the blood brain barrier (25-27).

The preceding observations speak to one of the central ideas of my invention, namely that the onset of plaque formation, plaque size or the number of plaques would be influenced by reducing levels of free beta-amyloid in the brain and these may in turn be linked to beta-amyloid levels in the blood. Beta-amyloid equilibria will be radically perturbed by sequestering or depleting soluble beta-amyloid in the blood using anti-beta-amyloid antibodies (Fig. 3). Induced endogenous antibodies or antibodies administered passively into the circulation can not cross the blood-brain barrier. Thus, they will act as a sink which should gradually reduce Aß levels in the brain by pulling the equilibrium to the right (Fig. 3).

Concentrations of antibody that are sustainable in animals would far exceed the levels of circulating beta-amyloid. For example, in both normal controls and patients with sporadic Alzheimer's disease, the plasma concentration of beta-amyloid was ~200 pM (6). For patients with familial Alzheimer's disease these levels were only 3-fold higher, 600 pM (6). The medium-range plasma level of a specific antibody in an immunized animal is ~0.150 mg/ml or 1,000,000 pM. Since this greatly surpasses plasma beta-amyloid levels, circulating beta-amyloid should be quantitatively bound by even low affinity antibodies. For example more then 99% of circulating beta-amyloid would be complexed by an antibody with an association constant of $10^8\ M^{-1}$.

The use of catalytic antibodies which can continuously destroy circulating beta-amyloid, would provide a highly efficient and permanent means of depleting these peptides in the blood (Fig. 4). This could provide an advantage over the use of

conventional antibodies which can bind beta-amyloid but do not destroy the peptide. Induced catalytic antibodies or catalytic antibodies administered passively into the circulation can not cross the blood-brain barrier. Thus, they will act as a sink which would gradually reduce beta-amyloid levels in the brain by pulling the equilibrium to the right (Fig. 4).

It might be desirable to deliver anti-beta-amyloid antibodies into the brain so that they are in immediate contact with both soluble and insoluble forms of the peptide. However, induced endogenous antibodies or antibodies administered passively into the peripheral circulation do not normally cross the blood-brain barrier. Blood-borne anti-beta-amyloid antibodies therefore, cannot reach beta-amyloid plaques or soluble beta-amyloid in the central nervous system. These peptide-specific antibodies can be directly infused into the brain or the intracerebroventricular space but this is usually a difficult proceedure. Fortunately, a universal method for the rapid, vector-mediated delivery of macromolecules across the blood-brain barrier has been devised (15-18). This system can be adapted to carry either conventional or catalytic anti-beta-amyloid antibodies over the blood-brain barrier so that they are in immediate contact with the beta-amyloid plaques and soluble beta-amyloid pool in the brain (Fig. 5).

A vector moiety must be chemically or genetically attached to the anti-beta-amyloid antibody to facilitate its delivery into the central nervous system. This vector component could be, for example, an anti-transferrin receptor or anti-insulin receptor antibody that binds to those receptors on the brain capillary endothelial wall (15-18) which makes up the blood-brain barrier. The resulting bifunctional antibody (28-32) will attach to appropriate receptors on the luminal side of the vessel (Fig. 5). Once bound to the receptor, both components of the bispecific antibody can pass across the blood-brain barrier by the process of transcytosis. Anti-beta-amyloid antibodies which have entered the brain will interact directly with both beta-amyloid plaques and the soluble beta-amyloid pool. It has been estimated that concentrations of macromolecules in the 10^{-8}-10^{-7}M range can be achieved in the brain using vector-mediated delivery (15).

Depending on their design, anti-beta-amyloid bispecific antibodies situated in the brain can function in three different ways to reduce soluble beta-amyloid and beta-amyloid deposits. An anti-beta-amyloid bispecific antibody that tightly binds soluble beta-amyloid will not only sequester the peptide but, due to efflux of vectorized molecules from the central nervous system (33), potentially can carry the bound beta-amyloid out of the brain and release it into the blood stream. This clearance mechanism would lead to a continuous cycling of beta-amyloid out of the brain (Fig. 6, path 1). The use of site-directed anti-beta-amyloid antibodies which can directly dissolve beta-amyloid aggregates (13) adds a new dimension to our strategy. Antibody-mediated dissaggregation of fibrilar beta-amyloid rendered it nontoxic *in vitro*. Importantly, a low ratio of antibody to beta-amyloid (1:10) was effective for this conversion (13) so that a similar effect could be achieved on the beta-amyloid plaques in the brain (Fig. 6, path 2). Another attractive possibility makes use of catalytic anti-beta-amyloid antibodies (9-12) designed to cleave beta-amyloid into harmless fragments. The advantage of delivering an beta-amyloid-specific catalytic antibody into the brain is two-fold. The beta-amyloid peptide would be permanently destroyed by such antibodies and, since catalysis is continuous, each antibody will inactivate many target beta-amyloid molecules in the brain (Fig. 6, path 3). Thus

much less vectorized bispecific antibody would have to be delivered into the central nervous system to achieve the desired depletion of beta-amyloid.

8) As far as I know there has been no prior solution to controlling beta-amyloid levels in the peripheral circulation or in the brain.

unpredictable

9) It requires that the inventor is aware both of the role that beta-amyloid plays in causing disease and realizes that antibodies can specifically sequester the beta-amyloid peptide which circulates in the body. It further requires that the inventor recognize that insoluble beta-amyloid is in a dynamic equilibrium with the soluble form and that specific antibodies can change that equilibrium. The potential for generating catalytic antibodies and for creating vectorized bispecific antibodies must also be realized by the inventor.

10) Different normal beta-amyloid peptides and transition-state peptide analogs have been synthesized in my laboratory. These represent the amino-terminal, middle and carboxyl-terminal regions of the beta-amyloid 43-mer. The transition-state analogs include phosphonate, statine, and reduced peptide bond modifications designed to elicit catalytic antibodies that will cleave beta-amyloid at different sites. Several beta-amyloid vaccines have been produced using these normal peptides and transition-state peptide analogs. Mice have been immunized with the vaccines and have produced either anti-beta-amyloid or anti-transition-state analog antibodies. Hybridoma clones have been established from the immunized mice and the monoclonal antibodies produced by these clones have been fully characterized. Vectorized bispecific antibodies have been constructed and tested for bifunctionality.

11) There are several advantages inherent in an immunological solution to the problem of controlling beta-amyloid levels in the body. Anti-beta-amyloid antibodies are highly specific reagents which will exclusively recognize and interact with beta-amyloid. They can be designed to bind beta-amyloid, dissolve its aggregates or catalytically cleave this peptide into harmless fragments. Antibodies are safe, highly compatible and designed by nature to function within the living organism. Specific anti-beta-amyloid vaccines can be designed to induce endogenous antibodies which are continuously present in the body of the vaccinated individual. The level of antibodies can be increased at any time by a simple booster injection of the vaccine.

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Summary

An Intellectual Property/Technology Transfer Administrator with hands-on experience in Biochemical Research. Proven track record in negotiating agreements for licensing technology, conducting literary and patent searches, designing and maintaining databases for agreements and research notebooks. Highly people oriented individual who works well independently and as part of a team.

Professional Experience

Alpha Beta Technology, Inc., Worcester, MA 1993-Present
Intellectual Property Associate 1993-Present
Responsible for all intellectual property: namely confidentiality agreements, research contracts and agreements, material transfer agreements, technology transfer, licensing agreements and patent estate. Reported directly to CEO.

- Negotiate agreements and term sheets for licensing of technology
- Work closely with inventors and outside patent council on US and foreign filings, office actions and prosecution.
- Conduct literary and patent searches for novelty and prior art.
- Maintain databases for research notebooks, manuscripts and agreements.
- Currently enrolled in Patent Resource Course to become Patent agent

Senior Research Associate I (3/95-2/97)
Member of the Biochemistry Group: responsible for research, development, design and execution of experiments that contribute to project strategy; as well as investigation, creation and development of new methods and technologies for project advancement.

- Functionally purified and characterized ß-glucanase from *P. pinophilum* culture and further characterized commercial glucan degrading enzymes
- Responsible for Quality Control assay measuring DNA content in products by PCR
- Member of Leukocyte Biology Group investigating the Betafectin® receptor
- Supervised and trained junior member of department

Research Associate II (3/93-3/95)

- Planned and conducted experiments for the development of analytical tools used in the characterization of glucanases
- Purification of enzymes by FPLC, BIOPILOT, ROTOFOR
- Assisted in antibody characterization by modifying and isolating substrates (GPC)

1988-1993 Center for Biomedical and Biophysical Science and Medicine, Harvard Medical School, Boston, MA
Research Assistant II (1990-1993)

- Responsible for biological and enzymatic activities of angiogenin
- Isolated and characterized tumor derived proteins
- Contributed in cloning the cDNA of a vascular permeability factor
- Trained junior technicians

Research Assistant I (1988-1990)

- Worked with biological assay and handling of small animals
- Operated HPLC and FPLC and cross trained in electrophoresis, ELISA, culture assays

EDUCATION

Emmanuel College, Boston, MA
B.S. Biochemistry, minor Biology, *cum laude* with distinction in field of study
Graduate Courses Harvard Extension School: Genetic Engineering
UMass Graduate School, Protein Structure and Mol. Mech. of Drug Action
Worcester Polytechnical Institute: Medicinal Chemistry

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Kevin M. Farrell

Shayne Y. Huff, Ph.D.
Technical Specialist*

Richard L. Sampson
Of Counsel

February 1, 1999

FACSIMILE COVER SHEET

FAXED 2/1/99

TO: Ms. Pamela Torpey

FAX NUMBER: (508) 476-9174

FROM: Kevin M. Farrell

SUBJECT: BBRI-2004
Immunological Control of Beta-Amyloid Levels

THERE WILL BE 1 PAGES INCLUDING THIS COVER SHEET.
CONFIRMATION WILL FOLLOW: YES ___ NO X

Receipt of the referenced new invention disclosure is hereby acknowledged.

Pamela,
Thank you for forwarding this disclosure. I will call you following our initial review –
K

AAFORMS\CORRESPO\FAXBLANK

Please Remit $180.00

=======

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Page: 1

Massachusetts Biotechnology Research Institute
20 Hampden Street
Roxbury MA 02119

February 28, 1999
Client No. BBRI-2004M

Attn: Ms. Sharon L. Prager

B-Amyloid Peptide

Fees

	Rate	Hours	
02/01/99 KMF studying new invention disclosure and acknowledging receipt of same;	225.00	2.20	495.00
02/05/99 SYH Studying new invention disclosure.	100.00	2.30	230.00
02/08/99 SYH Continuing review of new invention disclosure.	100.00	0.90	90.00
02/23/99 SYH Studying new invention disclosure; conferring with inventor (VR, SYH) Re: patentability - obviousness and novelty.	100.00	2.20	220.00
		----	--------
For Current Services Rendered		7.60	1,035.00

Expenses

02/01/99 Facsimile expense	1.00

Total Expenses	1.00
Total Current Work	1,036.00

MEMORANDUM

To: Disclosure file BBRI-2004: Immunological Approaches to Controlling Beta-Amyloid Levels in the Body
From: SYH SH
Date: February 8, 1999
Re: Initial Review of New Invention Disclosure for Patentability

---

The file contains three grant applications, two of which contain preliminary data which is significant (data pages of grant applications are flagged). If this preliminary data has not yet been publicly disclosed, it should be included in the patent application, and we should consider filing a non-provisional application instead of a provisional, and then filing a CIP upon generation of further results. Whether any of the data has been publicly disclosed should be discussed with the inventor.

Upon review of the Invention Disclosure Form, some question remains regarding obviousness of the invention. Under section (9) which describes why the invention is not obvious to one of skill in the art, the inventor lists several pieces of information which were required to make the invention, some of which may have been known to those of skill in the art at the time of the invention. This topic should be discussed further with the inventor prior to preparation of the application. There do not appear to be any novelty issues.

BBRI\CURR\2004.M1

Review for patentability (data?) if possible, draft a few claims.

Conferring w/ Inventor - Victor ~~Rosso~~ Raso

7/23/99

Q's:

1) Has any of the data contained w/in the 3 grant applications been publically disclosed? No.

Regarding why the invention isn't obvious to one of average skill in the art:

2). The invention disclosure that you submitted lists several ~~reasons why~~ pieces of info required to make the invention. ~~Have this info. been publicized~~ Is this information available to one of skill in the art?

[eg: antibodies can specifically sequester the B-amyloid peptide which circulate in the body.]

or was this information discovered by ~~R~~ you and kept undisclosed?

Other possible arguements against obviousness:

- Key pieces of info. necessary to make your invention that ~~were~~ are not available to the public? (e.g. catalytic Abs made to xsition state analog

- Potential caveats regarding the research - reasons why certain experiments might not have worked -

No one has used the Ab's in diseased animals = therapeutic effects - data w/in a year? Maybe

Sink effect:

Ab binding B-amyloid - entering brain → relying on equilibrium to draw away from brain.

MEMORANDUM

To: Disclosure file BBRI-2004: Immunological Approaches to Controlling Beta-Amyloid Levels in the Body
From: SYH SH
Date: February 23, 1999
Re: Discussion of Patentability/Obviousness of New Invention with Inventor

---

In a telephone conversation with Dr. Victor Raso, I discussed issues of public disclosure of the invention, prior art, and obviousness regarding the above referenced invention. Dr. Raso advised me that none of the data contained within the three grant applications in the New Invention Disclosure file had been publicly disclosed. Regarding the information presented as reasons the invention would not have been obvious to one of skill in the art, Dr. Raso informed me that the pieces of information listed under item (9) of the Invention Disclosure Form had been publicly disclosed by others. Bearing this in mind, they would not serve as good arguments against obviousness.

In a discussion with Dr. Raso regarding other possible arguments for non-obviousness, he informed me that the effect of antibody binding β-amyloid and keeping it in the blood, to permit less transmittal to organs and the brain was a somewhat unpredictable result. He stressed that the ability of such sequestration to have a sink effect in drawing β-amyloid away from the brain is an unpredictable outcome. However, experiments which demonstrate this are currently underway. Other non-obvious aspects of his invention which he informed me of also involve experiments which are underway. One example is whether sequestration of β-amyloid in the blood and/or a sink effect will have significant therapeutic effects. In addition, the results of future experiments with bispecific antibodies which cross the blood brain barrier and sequester β-amyloid are unpredictable regarding whether enough antibody will be transported to the brain to have a therapeutic effect. These arguments may be of use in the future upon securing intellectual property rights for future inventions relating to this work. I asked Dr. Raso to give some thought to other potential reasons why the current invention would not have been obvious to one of skill in the art, specifically focusing on reasons why one of skill in the art would not have predicted the results with a reasonable degree of certainty. He said he would give this some thought and then contact me later.

BBRI\CURR\2004.M2

Boston Biomedical Research Institute
20 Staniford Street
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Voice: (617) - 912-0300, *Ext.* 316

FAX COVER SHEET

DATE: 2/26/99

TO: Shane Huff

Fax No: 207-363-0528

FROM: Vic Raso

Number of pages including cover sheet: 2

Message:

Hope this helps on the "obvious concerns"

Vic

Vic Raso

9) **(Expanded)** Our approach to controlling beta amyloid levels in the body has not been obvious for several reasons. These include: 1) Potential Safety Concerns; 2) Possible Inaccessibility to Beta-Amyloid in Plaques; 3) Unawareness of the Stoichiometric Balance; and 4) Lack of Related Models.

1) Potential Safety Concerns; The beta-amyloid peptide and amyloid precursor protein are naturally occurring molecules that are present in the body. Many researchers have therefore assumed that the simultaneous presence of anti-beta-amyloid antibodies would be harmful. In contrast, I found that normal animals immunized with beta-amyloid antigens and animals bearing ascites which produce large amounts of anti-beta-amyloid antibodies are not adversely affected. Thus the continual presence of anti-beta-amyloid antibodies which can bind to both beta-amyloid and to the amyloid precursor protein apparently does not interfere with the normal functioning of the animal.

2) Possible Inaccessibility of Beta-Amyloid in Plaques; Most scientists assume that to be effective, anti-beta-amyloid antibodies must react directly with the beta-amyloid that is aggregated within the amyloid plaque. Antibodies may not have access to this aggregated form of the peptide and therefore would not be able to dissolve the plaque. I realized that this is an incorrect assumption and that in order to dissolve the plaque, anti-beta-amyloid antibodies would only have to react with the low-level of soluble beta-amyloid which is in equilibrium with the aggregate. The plaque will be gradually disassembled as the outside layers of dissociated beta-amyloid molecules are prevented from re-assembling onto the aggregate by the presence of the anti-beta-amyloid antibodies which will rapidly bind to them.

3) Unawareness of the Stoichiometric Balance; It is not inherently obvious that there will be a very favorable stoichiometry between the levels of soluble beta-amyloid and anti-beta-amyloid antibodies in an immunized subject. I performed the calculations based upon reported concentrations of beta-amyloid found in the blood stream and the concentration of anti-beta-amyloid antibodies expected following immunization with beta-amyloid antigens. The results indicate that the anti-beta-amyloid antibodies will be in a 1000-2000-fold excess over beta-amyloid and that greater than 99% of the circulating beta-amyloid will be complexed by the antibodies in an immunized individual.

4) Lack of Related Models; Until recently there have been very few good examples for therapeutically using monoclonal antibodies in diseased patients. We selected antibodies to target beta-amyloid because they are compatible, highly specific molecules that are designed by nature to recognize and seek-out pathogens in the body. Currently, a humanized anti-receptor monoclonal antibody, Herceptin, is giving positive signs in the treatment of breast cancer. The ability of this passively administered antibody to produce a beneficial response in the face of all of the potential physiological hurdles posed by the disease is very promising. My approach for controlling beta-amyloid levels could similarly be used to develop an immunotherapeutic approach to the safe treatment of Alzheimer's disease.

207-363-0528

Farrell & Associates, P.C.
12 Riverwood Drive
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York Harbor, Maine 03911

March 31, 1999
Client No. BBRI-2004M

Boston Biomedical Research Institute
P.O. Box 981
Douglas MA 01516

Attn: Ms. Pamela Torpey

B-Amyloid Peptide

Previous Balance $1,036.00

Fees

Date		Description	Rate	Hours	Amount
02/26/99	KMF	studying correspondence from Dr. Raso relating to obviousness issues; studying disclosure and conferring (SYH, KMF);	225.00	1.80	405.00
03/01/99	SYH	Studying fax received from inventor (VR) re: arguments against obviousness; beginning drafting claims.	100.00	2.00	200.00
	KMF	conferring (SYH, KMF) regarding claims drafting;	225.00	0.50	112.50
03/02/99	SYH	Conferring with inventor Re: arguments for non-obviousness of invention; continuing drafting claims.	100.00	7.00	700.00
03/03/99	SYH	Studying materials transmitted by inventor (VR) Re: new invention; continuing drafting claims.	100.00	4.50	450.00
03/04/99	SYH	Beginning drafting Exemplification section of Patent Application.	100.00	6.50	650.00
03/08/99	SYH	Continuing drafting of Exemplification from two grant			

proposals provided.	100.00	6.50	650.00

03/09/99

SYH Continuing drafting and revising

B-Amyloid Peptide

Date	Attorney	Description	Rate	Hours	Amount
		of Exemplification section; incorporating new material received from inventor into claims.	100.00	7.00	700.00
03/10/99	SYH	Editing claims; studying requested prior art Re: patentability of claims as drafted.	100.00	3.20	320.00
	KMF	studying draft Claims and Exemplification sections; conferring (SYH, KMF) regarding same;	225.00	1.60	360.00
03/17/99	SYH	Reviewing inventor's (VR) comments Re: claims; revising claims according to inventor comments; transmitting information to and requesting additional information from inventor (VR).	100.00	3.00	300.00
03/18/99	SYH	Editing Exemplification; transmitting Exemplification to inventor (VR); beginning drafting Detailed Description of invention.	100.00	7.00	700.00
03/19/99	SYH	Continuing drafting Detailed Description of the invention.	100.00	7.00	700.00
03/22/99	SYH	Continuing drafting Detailed Description; drafting Background of the Invention.	100.00	6.80	680.00
03/23/99	SYH	Editing Detailed Description of the invention.	100.00	0.90	90.00
03/24/99	SYH	Conferring with inventor (VR, SYH) re: Methods of the invention, additional claims, and results presented in			

Exemplification.	100.00	0.80	80.00

03/25/99
SYH Revising Detailed Description of

Boston Biomedical Research Institute — March 31, 1999 — Client No. BBRI-2004M

B-Amyloid Peptide

		Rate	Hours	
	the invention as per new materials received from inventor (VR).	100.00	1.40	140.00
03/29/99 SYH	Continuing revising Detailed Description re: new material transmitted from inventor (VR).	100.00	0.60	60.00
03/31/99 SYH	Requesting information from inventor (VR).	100.00	0.20	20.00
			-----	--------
	For Current Services Rendered		68.30	7,317.50

Expenses

03/02/99	Facsimile expense	1.00
03/02/99	Facsimile expense	5.00
03/10/99	Facsimile expense	8.00
03/18/99	Facsimile expense	42.00
03/29/99	Document Supply Expense - ordering references via agent;	30.00

	Total Expenses	86.00
	Total Current Work	7,403.50
	Balance Due	$8,439.50
		=========

Aged Due Amounts

0-30	31-60	61-90	91-120	121-180	181+
7,403.50	1,036.00	0.00	0.00	0.00	0.00

Please Remit $8,439.50

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Richard L. Sampson
Of Counsel

March 2, 1999

FACSIMILE COVER SHEET

FAXED 3/2/99

TO: Dr. Vic Raso

FAX NUMBER: (617) 912-0308

FROM: Shayne Huff SH

SUBJECT: β-Amyloid Directed Immunotherapy
Our Reference No.: BBRI-2004

THERE WILL BE 5 PAGES INCLUDING THIS COVER SHEET.
CONFIRMATION WILL FOLLOW: YES ___ NO X

Dear Dr. Raso:

Following are the first page and specific aims page from two of the three grant applications we have in our file. As discussed on the phone, please send an electronic copy of the corresponding grant applications as attachments in MS word. I won't need an electronic copy of the third application which contains no data. My e-mail address is SYHuff@aol.com. I'll begin drafting claims which specifically recite the invention(s) and will send them off for your comments in a few days.

Best regards,

Shayne

A:\FORMS\CORRESPO\RASO.FAX

Grant 1

DESCRIPTION. State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This description is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. DO NOT EXCEED THE SPACE PROVIDED.

The ß-amyloid peptide and the cerebral plaques that it forms are likely either the direct or indirect cause of Alzheimer's disease. This small, 43 amino acid residue peptide is produced in the central nervous system by cleavage from a cell-surface precursor protein. Thus, soluble ß-amyloid exists free in the brain and cerebrospinal fluid and it eventually deposits as "insoluble" aggregates to form amyloid plaques in the brain. The hypothesis to be tested suggests that the soluble and insoluble forms of ß-amyloid present within the brain of Alzheimer's patients are in dynamic equilbrium. Accordingly, plaque growth will be curtailed and the plaques should gradually dissolve when that equilibrium is displaced by reducing soluble ß-amyloid levels. This depletion can be accomplished by using highly specific anti-ß-amyloid antibodies to tie up or modify soluble ß-amyloid in the brain. Transport of the anti-ß-amyloid antibody into the central nervous system will be facilitated by coupling it to an anti-transferrin receptor antibody which serves as a vector, carrying the new bispecific construct across the blood-brain barrier. These expressly designed, vectorized anti-ß-amyloid antibodies provide a novel basis for the immunotherapy of Alzheimer's disease by virtue of their ability to enter the brain and directly perturb ß-amyloid equilibria.

The effect of those vectorized antibodies will be tested using an established colony of TS2576 transgenic mice which express a mutant form of the human amyloid precursor protein and produce extracellular ß-amyloid peptide deposits in the brain at ~11 months of age. Human ß-amyloid peptides and transition state analogs of those peptides have been used to elicit monoclonal antibodies in mice. The antibodies have been chacterized by ELISA and proteolytic assays to select those that show high-affinity binding to ß-amyloid, or catalytic activity, or an ability to dissolve ß-amyloid aggregates. Bispecific antibodies were formed by coupling the anti-ß-amyloid antibodies to an anti-transferrin receptor antibody which can cross the blood-brain barrier by transcytosis. These vectorized bispecific antibodies will be administered to young and old transgenic mice by periodic i.p. injection either before or after the onset of plaque development. Antibody-treated transgenic mice will be compared to control transgenic mice in terms of the number and size of plaques in brain sections, the level of ß-amyloid peptides in brain extracts and the extent of their memory impairment. These studies could establish a causal relationship between amyloid deposits and the behavioral deficits in the transgenic mouse model.

PERFORMANCE SITE(S) *(organization, city, state)*

BOSTON BIOMEDICAL RESEARCH INST
20 STANIFORD STREET
BOSTON, MA 02114

KEY PERSONNEL. See instructions on Page 11. *Use continuation pages as needed* to provide the required information in the format shown below.

Name	Organization	Role on Project
Victor Raso	Boston Biomedical Research Institute	Principal Investigator
Katherine Sheldon	Boston Biomedical Research Institute	Research Associate
Christine Kearney	Boston Biomedical Research Institute	Research Technician

Grant 1

A. SPECIFIC AIMS

Presently, there are few encouraging therapeutic prospects for the prevention or treatment of Alzheimer's disease. However, the reversable deposit of soluble ß-amyloid peptide (Aß) onto plaques in unfixed brain sections and the disintegration of Aß aggregates by monoclonal antibodies suggest that an immunotherapeutic approach to the disease should be feasible. The ultimate objective of this project is to test four central hypotheses which would lay the scientific groundwork for such a therapy. These are: 1) that ß-amyloid plaques in the brain are in equilibrium with soluble Aß peptide; 2) that anti-Aß antibodies can be carried through the blood-brain barrier and into the central nervous system by coupling them to specific vector antibodies; 3) that soluble Aß will be sequestered or depleted by appropriate anti-Aß antibodies in the brain; and 4) that decreasing soluble Aß levels in the central nervous system will either reduce the number or size of preestablished ß-amyloid plaques and/or prevent the *de novo* formation of incipient plaques.

The advantages inherent in the immunotherapy of disease are well established. No harmful drugs or foreign proteins are required for treatment. Immunotherapy is highly specific and can be easily sustained for as long as needed, important features for combating Alzheimer's disease. An ability to experimentally modulate plaque formation in the brain would allow us to unequivocally test the causal relationship between these lesions and memory impairment in affected animals. Our rational assembly of reagents, techniques and model system currently puts us in a unique position to establish the scientific basis for this novel and potentially high impact approach to treating Alzheimer's disease.

The three major research aims are to:

A.1 Elicit Monoclonal Antibodies with Native Aß and Transition State Aß Antigens

a. Immunize mice with several different native Aß and transition state Aß antigens and establish antibody-producing hybridoma clones.

b. Screen and isolate antibodies that dissaggregate Aß or show catalytic activity or have a high binding affinity for the Aß peptide.

A.2 Produce Vectorized Anti-Aß/Anti-Receptor Bispecific Antibodies

a. Covalently-link selected anti-Aß antibodies with anti-receptor antibodies to produce bifunctional heterodimers which readily cross the blood-brain barrier.

b. Prepare small bispecific $F(ab')_2$ heterodimers for vector-mediated transport into the brain without the danger of complement fixation.

c. Characterize these bifunctional reagents with respect to their dual specificity and their ability to enter the brain.

A.3 Test Anti-Aß Bispecific Antibodies for Disrupting Plaque Development in Mice

a. Determine if the vectorized bispecific anti-Aß antibodies cross the blood/brain barrier and bind to amyloid plaques found in the brain of "Alzheimer's mice."

b. Treat young "Alzheimer's mice" with vectorized bispecific anti-Aß antibodies and examine the animals for effects on subsequent amyloid plaque development and for the prevention of behavioral deficits.

c. Treat old, plaque-bearing "Alzheimer's mice" with vectorized bispecific anti-Aß antibodies and examine the animals for a reduction in the number or size of preestablished amyloid plaques and reversal of their memory impairment.

Grant 2

DESCRIPTION. State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This description is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. DO NOT EXCEED THE SPACE PROVIDED.

The long-term objective of this project is to develop a new innovative approach for the treatment and/or prevention of Alzheimer's disease. It is based upon basic immunological principles and uses vaccine methodology to produce peptide-specific antibodies designed to sequester systemic ß-amyloid. The scientific concepts established in this research have potential for broad biomedical impact since they might be applicable to other similar diseases or conditions where an offending substance must be selectively removed, sequestered or redistributed in the body.

The ß-amyloid peptide and the cerebral plaques that it forms are likely either the direct or indirect cause of Alzheimer's disease. This peptide is produced in both the brain and peripheral tissues by cleavage from a common cell-surface precursor protein. Soluble ß-amyloid exists free in the blood and cerebrospinal fluid while "insoluble" aggregates are deposited in the brain as amyloid plaques. The soluble and insoluble forms of ß-amyloid present within Alzheimer's patients appear to be in dynamic equilibrium. This equilibrium will be displaced away from the brain by developing a vaccine designed to generate peptide-specific antibodies in a transgenic mouse model of Alzheimer's disease. Restricted to the peripheral circulation, these antibodies will sequester ß-amyloid peptide in the blood and by doing so could gradually deplete intercommunicating peptide levels in the brain. Decreased concentrations of ß-amyloid in the brain should reduce the size and number of brain plaques or delay their appearance.

These studies would establish whether or not there is a causal relationship between ß-amyloid deposits and memory impairment in these transgenic mice. Moreover, this expressly designed vaccine and the anti-ß-amyloid antibodies it elicits could produce beneficial therapeutic effects by perturbing critical ß-amyloid equilibria in the mouse model. If successful this research using ß-amyloid vaccines would establish a scientific basis for the specific immunotherapy of Alzheimer's disease and other similar conditions.

PERFORMANCE SITE(S) (organization, city, state)

BOSTON BIOMEDICAL RESEARCH INST
20 STANIFORD STREET
BOSTON, MA 02114

KEY PERSONNEL. See instructions on Page 11. Use continuation pages as needed to provide the required information in the format shown below.

Name	Organization	Role on Project
Victor Raso	Boston Biomedical Research Institute	Principal Investigator
Christine Kearney	Boston Biomedical Research Institute	Research Technician

A. SPECIFIC AIMS

Presently, there are few encouraging therapeutic prospects for the prevention or treatment of Alzheimer's disease. However, the reversable deposit of soluble ß-amyloid peptide (Aß) onto plaques in unfixed brain sections and the disintegration of Aß aggregates by monoclonal antibodies suggest that an immunotherapeutic approach to the disease should be feasible. The ultimate objective of this project is to develop novel therapeutic vaccines which will elicit specific anti-Aß antibodies. By efficiently binding Aß in the blood stream these antibodies should perterb its systemic equilibrium, gradually deplete Aß levels in the brain and thus delay or reverse plaque formation. This new, innovative form of intervention might ultimately be used to prevent or alter the course of Alzheimer's disease.

The advantages inherent in the immunotherapy of disease are well established. No harmful drugs or foreign proteins are required for treatment. Immunotherapy is highly specific and can be easily sustained for as long as needed, important features for combating Alzheimer's disease. In order to achieve our goal we will apply basic principles from the field of immunology and from the newly expanding field of vaccine design and development. An ability to experimentally modulate plaque formation in the brain would allow us to unequivocally test the causal relationship between these lesions and memory impairment in affected animals. Moreover, the new paradigms established in this study have potential for a broad biomedical impact since they might easily apply to alternative situations where low levels of a noxious substance cause or initiate disease.

Our rational assembly of reagents, techniques and model system currently puts us in a unique position to test the scientific feasibility of this novel and potentially high impact approach to Alzheimer's disease. This study will use Aß peptides to create vaccines designed to induce anti-Aß antibodies which will alter systemic Aß levels. We can then test these reagents for beneficial effects in a transgenic mouse model of Alzheimer's disease. Our research will establish whether there is potential for the clinical development of anti-Aß vaccines as uniquely specific and urgently needed therapeutics.

The three major research aims are to:

A.1 Produce ß-Amyloid Peptide Vaccines

a. Synthesize several small Aß peptides encompassing different regions of the complete 43 amino acid residue Aß peptide.

b. Assemble ß-amyloid peptide vaccines for immunization by disulfide-linking the various Aß peptides to a keyhole limpet hemocyanin carrier protein.

A.2 Immunize Transgenic Mice with Aß Peptide Vaccines

a. Immunize groups of both young and old transgenic "Alzheimer's mice" with the different Aß peptide vaccines to establish high titre anti-Aß antibody production.

b. Screen sera of immunized mice using a radiolabeled-Aß binding assay and ELISA to characterize both the strength and specificity of the antibody response.

A.3 Test Vaccines for Disrupting Plaque Development in Mice

a. Determine if endogenous antibody binding of systemic Aß alters the biodistribution of radiolabeled Aß in "Alzheimer's mice."

b. Examine young vaccinated "Alzheimer's mice" for an effect of endogenous antibodies on the de novo formation of amyloid plaques and for the prevention of memory impairment.

c. Test the effect of vaccination on old, plaque-bearing "Alzheimer's mice." Examine the animals for a reduction in the number or size of preestablished amyloid plaques and reversal of their behavioral deficits.

Boston Biomedical Research Institute
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Voice: (617) - 912-0300, *Ext.* 316

FAX COVER SHEET

DATE: 3/5/99

TO: Shyne Huff

Fax No: 207-363 0528

FROM: Vic Raso

Number of pages including cover sheet:

Message:

201-96?-0558

Raso, Victor A

C. PRELIMINARY STUDIES

C.1 Elicit Monoclonal Antibodies with Native Aß and Transition State Aß Antigens

a. Synthesis of ß-amyloid peptide antigens: The amino acid sequence of the 43 residue ß-amyloid peptide (Aß) is shown in Fig. 3. Predicting precisely which site on the Aß peptide will be ultimately best suited for antibody-mediated therapy is difficult. Therefore 3 key epitopes on the

H_2N-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIAT-CO_2H

1 10 20 30 40

(Fig. 1) ~~Fig. 3~~

Aß 43-mer were chosen as targets for the catalytic and conventional antibodies. The resulting panel of monoclonal antibodies will be screened *in vitro* to identify desirable properties such as high affinity binding, catalytic activity and/or the ability to dissociate Aß aggregates. Select anti-Aß antibodies will then be vectorized by coupling to an anti-transferrin receptor antibody (anti-TfR) and studied in the Tg mouse model to determine empirically which of these unique immuno-reagents are both therapeutically effective and pharmacologically safe.

Peptide antigens for eliciting an immune response directed against the amino-terminus, the central region and the carboxy-terminus of Aß were produced (Fig. 4). A Cys residue was added and the peptides were synthesized using standard automated Fmoc chemistry. The peptides were purified by HPLC and their composition was verified by mass spectral and amino acid analysis. The Cys substitution was designed to provide a sulfhydryl linkage group for coupling the peptides to antigenic, maleimide-activated carrier proteins such as Keyhole Limpet Hemocyanin (KLH).

Fig 2 H_2N-Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Cys-amide

1 5 10 15

Fig 3 Cys-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-amide

10 15 20 25

Fig 4 Cys-Met-Val-Gly-Gly-Val-Val-Ile-Ala-Thr-CO_2H

~~Fig. 4~~ 35 40

b. Phosphonamidate and phosphonate based transition state peptides: A phosphonamidate transition state analog encompassing the carboxy-terminal region of Aß has been synthesized (Fig. 5).

↓

N-acetyl-Cys-Met-Val-Gly-Gly--**CO-NH**--Val-Val-Ile-Ala-amide

35 40

Fig 10 ~~Fig. 5~~ N-acetyl-Cys-Met-Val-Gly-Gly-**PO_2^--NH**-Val-Val-Ile-Ala-amide

Replacement of the proposed scissile peptide linkage between Gly_{38} and Val_{39} (↓) with a phosphonamidate moiety (**-PO_2^--NH-**) is designed to elicit catalytic antibodies that will hydrolytically cleave Aß at this site. The N-acetyl-Cys residue was placed at the position of Leu_{34} to provide

a suitable linkage group for coupling this peptide to an antigenic carrier protein. The structures shown in Fig. 6 represent the putative transition state for peptide hydrolysis by zinc peptidases and the phosphonate and phosphonamidate mimics. Similar tetrahedral transition state

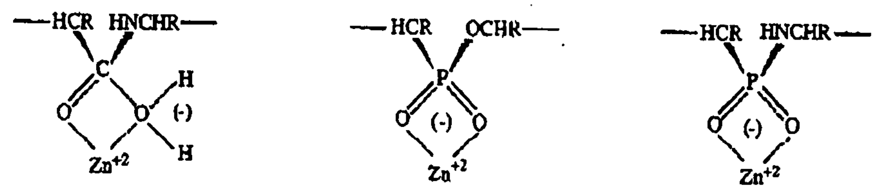


Fig. 6 Transition-state Phosphonate Phosphonamidate

intermediates are formed in each of the four classes of proteolytic enzymes, the serine-, cysteine-, aspartic- and metallo-peptidases.

The design strategy and methods for synthesizing phosphonamidate based transition state peptides are straightforward (46, 47). The N-terminal portion of the peptide (N-acetyl-Cys-Met-Val-Gly) was made using standard automated Fmoc chemistry. Its amino terminus was capped with acetic anhydride while it was still attached to the resin. After cleavage from the resin the N-acetyl tetrapeptide was treated with pyridine disulfide to protect its sulfhydryl group. An acid chloride of Cbz-glycine phosphonate monomethyl ester (46, 47) was coupled with Val-Val-Ile-Ala-amide which was synthesized by automated Fmoc chemistry. The last amino acid of Aß, Thr, was omitted because of potential problems with its unprotected hydroxyl group. The product, Cbz-Gly-PO_2^--NH Val Val Ile-Ala-amide has a phosphonamidate (methyl ester) bond between the Gly and Val residues. Next, the Cbz blocking group was removed using hydrogen so that the protected N-acetyl-Cys-Met-Val-Gly peptide could be added to the amino terminal end of this transition state peptide by HBTU-activated peptide linkage. Treatment with mercaptoethanol and rabbit liver esterase was used to deblock the peptide. Each key component listed in the synthetic scheme was tested for purity by HPLC and its composition was verified by mass spectral and amino acid analysis. This new Aß analog, N-acetyl-Cys-Met-Val-Gly-Gly-**PO_2^--NH**-Val-Val-Ile-Ala-amide (Fig. 5) is designed to elicit catalytic antibodies that will specifically cleave Aß at the Gly-Val bond.

The synthesis of phosphonate Aß transition state peptides (eg. N-acetyl-Cys-Met-Val-Gly-Gly-**PO_2^--O**-Val-Val-Ile-Ala-amide) will follow a similar scheme and will use some of the same intermediates described for the phosphonamidate transition state analog.

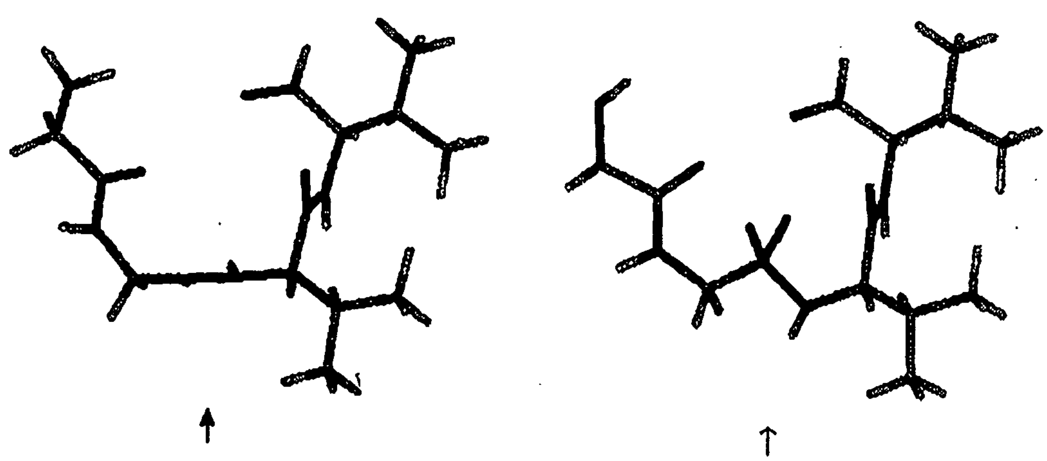


Fig 12?

~~Fig 7~~ Gly-Gly-**CO-NH**-Val-Val Gly-Gly-**PO_2^--NH**-Val-Val

A structural comparison was made between the native Aß peptide and the transition state phosphonamidate Aß peptide (Fig. 7) using a graphics workstation. The peptide link **-CO-NH-** (↑) between Gly_{38} and Val_{39} was replaced with a phosphonamidate bond **-PO_2^--NH-** (↑) and an energy minimization was applied. The orientation shown ~~above~~, Fig. 7, clearly illustrates the difference

between the planar peptide link **-CO-NH-** (↑) of native Aß on the left versus the corresponding tetrahedral phosphonamidate bond **-PO_2^--NH-** (↑) in the transition state peptide on the right.

An antibody combining site complementary to the tetrahedral transition state analog on the right of Fig. 7, will force the normally planar bond of the Aß substrate peptide on the left into a transition state-like conformation. Such bond distortion can catalyze the hydrolytic cleavage of the Aß peptide at the Gly_{38}-Val_{39} linkage.

c. Statine based transition state peptides: A series of statine (**Sta**) transition state analogs encompassing the carboxy-terminal region of Aß (Cys-Met-Val-Gly-Gly-Val/**Sta**-Val/**Sta**-Ile/**Sta**-Ala-Thr) has been synthesized in this laboratory (Fig. 8).

↓ ↓ ↓

Met-Val-Gly-Gly-Val-Val-Ile-Ala-Thr-CO_2H

35 40

Fig. 6

~~Fig. 8~~

Replacement of the proposed scissile peptide linkage between Val_{39} and Val_{40} (↓), Val_{40} and Ile_{41} (↓) and Ile_{41} and Ala_{42} (↓) with a "statyl" moiety (-CHOH-CH_2-CO-NH-) is designed to elicit catalytic antibodies that will hydrolytically cleave Aß at one of these sites. A Cys residue was placed at the position of Leu_{34} (Fig. 3) to provide a suitable linkage group for coupling this peptide to a maleimide-activated carrier protein.

The statine transition state peptides were entirely synthesized using standard automated Fmoc chemisty. This was feasible due to the availability of Fmoc-statine (**Sta**), [N-Fmoc-(3S,4S)-4-amino-3-hydroxy-6-methyl heptanoic acid] from a commercial source. Each peptide was tested for purity by HPLC and its composition was verified by mass spectral and amino acid analysis.

d. Phenylalanine statine based transition state peptides: A series of phenylalanine statine (**PhSta**) transition state analogs encompassing the central region of Aß (Cys Tyr Glu-Val-His-His-Gln-Lys-Leu-Val-Phe/**PhSta**-Phe/**PhSta**-Ala-Glu-Asp-Val-Gly-amide) was synthesized in this laboratory (Fig. 9).

↓ ↓

Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-amide

10 15 20 25

Fig. 7

~~Fig. 9~~

Replacement of the proposed scissile peptide linkage between Phe_{19} and Phe_{20} (↓) and Phe_{20} and Ala_{21}(↓) with a statyl moiety (**-CHOH-CH_2-CO-NH-**) (Fig. 9) is designed to elicit catalytic antibodies that will hydrolytically cleave Aß at these sites (Fig. 9). A Cys residue was placed at the position of Gly_9 (Fig. 3) to provide a sulfhydryl linkage group for coupling the peptide to antigenic, maleimide-activated carrier proteins such as KLH.

The phenylalanine statine transition state peptides were entirely synthesized using standard automated Fmoc chemisty. This was feasible due to the recent availability of Fmoc-"phenylalanine statine" (**PhSta**), [N-Fmoc-(3S,4S)-4-amino-3-hydroxy-5-phenylpentanoic acid] from a commercial source. Each peptide was tested for purity by HPLC and its composition was verified by mass spectral and amino acid analysis.

An antibody combining site that is complementary to the elongated tetrahedral transition state analog will force the normally planar peptide bond of the Aß substrate into a transition state-like conformation. Such distortion should catalyze the cleavage of Aß at the Phe_{19}-Phe_{20} bond.

e. Immunization of mice: Standard protocols were used to immunize BALB/c mice with the KLH-linked Aβ peptides described in the preceding sections. Briefly this procedure used i.p. injection of the different antigens emulsified in complete Freunds adjuvant, followed by a second course in incomplete Freunds adjuvant. Three days prior to the hybridoma fusion, the mice were boosted i.v. with antigen in PBS.

f. ^{125}I-Aβ binding assay: It was very important to demonstrate that our anti-Aβ and anti-transition state Aβ monoclonal antibodies bind to the natural $A\beta_{1-43}$ peptide which they are designed to cleave. Therefore we radiolabeled $A\beta_{1-40}$ and $A\beta_{1-43}$ with ^{125}I and then separated the iodinated peptide from unlabeled material by HPLC to give essentially quantitative specific activity (~2000 Ci/mmol) (13). This probe was incubated for 1h at 23°C with either purified anti-Aβ antibodies or media taken from hybridoma clones producing anti-Aβ antibodies. A polyethylene glycol separation method was used to detect the amount of ^{125}I-$A\beta_{1-43}$ bound to antibody (Table I).

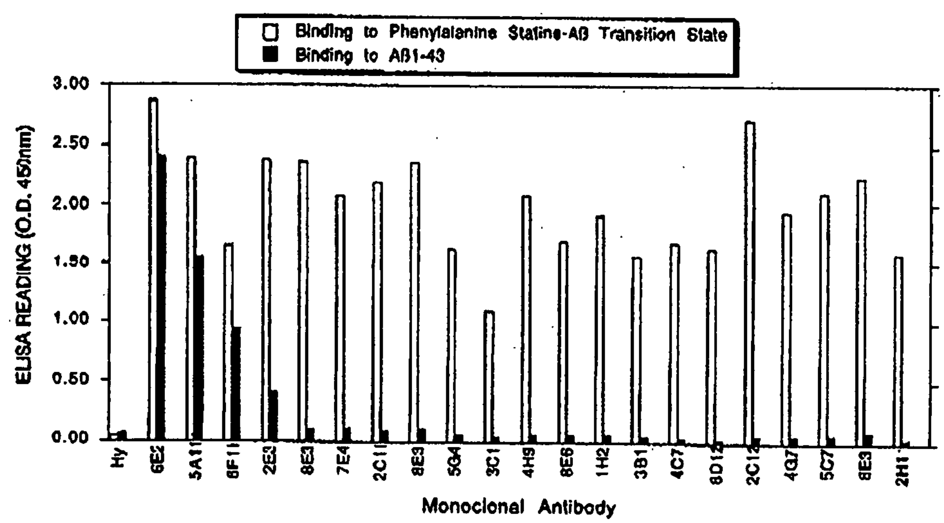
Table I **^{125}I-$A\beta_{1-40}$ Binding to a Purified Monoclonal Anti-Aβ Antibody ***

Addition	^{125}I-$A\beta_{1-40}$ Bound (cpm)	Specifically Bound (% of total added)
Control	8,560	-
+ 5A11 anti-Aβ	64,589	79

* anti-Aβ 5A11 at 2×10^{-6} M; Added ~70,000 cpm of ^{125}I-$A\beta_{1-40}$

The data in Table I demonstrate the ability of our purified 5A11 monoclonal anti-Aβ antibody to bind a high percent of ^{125}I-$A\beta_{1-40}$. This binding assay will be valuable to screen clones and purified antibodies (Table I) for their ability to bind Aβ and can also serve as the basis for a competitive displacement assay to measure the relative binding strength of different unlabeled Aβ peptides. With very efficient catalytic antibodies this binding assay may have to be performed on ice to ensure that no cleavage of Aβ occurs during the 1h incubation time. The assay will allow us to quickly identify clones which produce high affinity anti-Aβ antibodies.

g. Hybridoma production I: We performed a hybridoma fusion using the spleen of a mouse immunized with the phenylalanine statine transition state Aβ-KLH antigen (Fig. 9). Monoclonal antibodies from several of the hundreds of hybridoma supernatants produced were screened using ELISA to assess their binding to both the normal $A\beta_{1-43}$ peptide and to the phenylalanine statine transition state Aβ peptide. Two major patterns were found (Fig. 10).



One group of antibodies (at the left of Fig. 10) bound to the immunizing transition state peptide and cross-reacted strongly with the native $A\beta_{1-43}$ peptide when each was adsorbed directly onto the ELISA plate. A second group (at the right) showed a high binding preference for the phenylalanine statine transition state Aß peptide and reacted minimally with native $A\beta_{1-43}$.

Strong color reactions were obtained in this ELISA using only 10 µl of hybridoma supernatent while Hy media alone or PBS gave a low background (Fig. 10). These results demonstrate that the comparative ELISA screen, although only a semi-quantitative measure of binding, will provide a means for choosing monoclonal antibodies that are highly selective for, and most reactive with, the transition state. Importantly, the antibodies bound to the carrier free Aß peptides adsorbed directly onto microtitre plates, showing their anti-peptide specificity.

These findings indicate that several of the new anti-Aß transition state antibodies are unique. They can bind to both the phenylalanine statine- and normal-Aß peptides. Their selective recognition of the transition state and weaker cross-reaction with native $A\beta_{1-43}$ however implies that this binding interaction is very different from that shown by conventional anti-native Aß antibodies. It suggests further that these new antibodies may be able to force the native Aß peptide into a conformation resembling the transition state for hydrolytic cleavage. Importantly, some of the antibodies which showed only minimal binding to $A\beta_{1-43}$ in this ELISA, did display cross-reactivity with the natural peptide using a highly sensitive ^{125}I-$A\beta_{1-43}$ binding assay (Table I).

h. Solid phase and TLC Aß proteolytic assays: A solid phase ^{125}I-labeled Aß assay was developed to screen anti-transition state antibody hybridoma supernatants for specific proteolytic activity. The Cys-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Tyr-amide peptide encompassing amino acids 14-25 of Aß (Fig. 3) was synthesized with a Cys and Tyr added at either end. This was radiolabeled with ^{125}I and the iodinated peptide was then separated from unlabeled material by HPLC to give essentially quantitative specific activity (~2000 Ci/mmol). The highly radioactive Aß peptide was coupled to a thiol-reactive, iodoacetyl-Sepharose gel to form an irreversible linkage. Catalytic antibodies should promote the progressive release of soluble ^{125}I-peptide from the solid phase matrix. The proposed assay was verified by the ability of several different proteases in to rapidly hydrolyze this Sepharose-linked Aß substrate. The peptide is readily accessible to proteolytic cleavage as revealed by the release of soluble ^{125}I-peptide that increased with incubation time (data not shown). Selected antibodies were screened for catalytic activity using release of radioactivity from ^{125}I-Aß-Sepharose (Fig. 11).

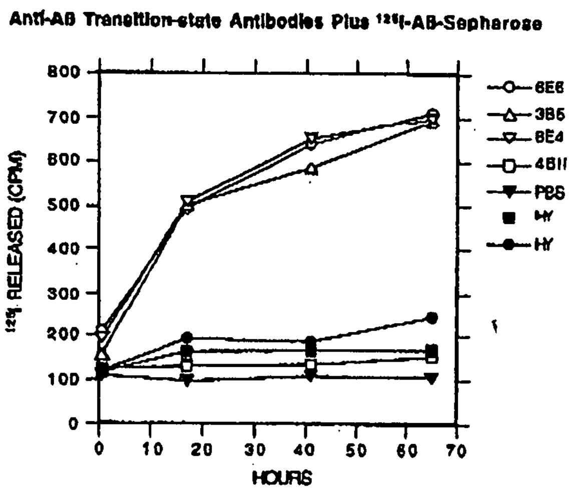


Fig. 11 Cleavage of ^{125}I-Aß-Sepharose

The results obtained at pH 7, 25°C indicate that the antibody-containing media of several clones released ^{125}I-peptide at a greater rate than other clones from this fusion or the PBS and Hy medium controls (Fig. 11). Large amounts of these antibodies will now be obtained, purified and tested a higher concentrations to achieve much faster rates of cleavage and to verify that the antibodies are acting in a catalytic mode using conventional enzyme kinetics. By changing the composition of the ^{125}I-peptide we can use this same strategy assay antibodies reactive with different regions of Aß.

We devised a thin layer chromatography-based autoradiography assay so that more definitive evidence for antibody-mediated cleavage of Aß could be obtained. We also expanded selected anti-phenylalanine statine Aß transition state clones, induced ascites production and isolated the different monoclonal antibodies using protein A-Sepharose. The cleavage assay used ^{125}I-$A\beta_{1-40}$ and a 17-mer, encompassing amino acids 9-25 (Fig 3). These two ^{125}I-labeled peptides bound to the purified monoclonal antibodies 5A11 and 6E2 when examined using either a PEG precipitation assay or by a co-electrophoresis method. To test for peptide cleavage we added the antibodies to the ^{125}I-peptides, allowed them to incubate and then spotted the reaction mix onto polyamide thin layer

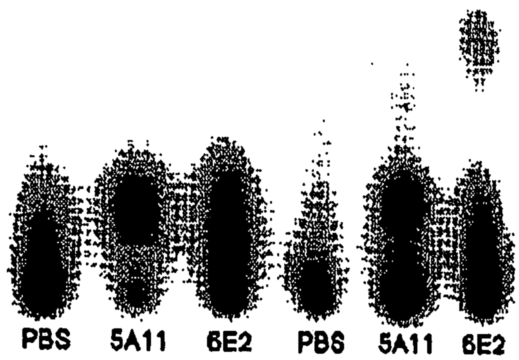


Fig. 12 TLC of ^{125}I-Aß-Cleavage

sheets. The chromatographs were developed in different solvents (eg. 0.5N HCl, 0.5N NaOH or pH7 phosphate buffer) and the migration of ^{125}I-products was followed by exposing the sheet using a quantitative phosphoimager system (Fig. 12).

It is very encouraging to see that these antibodies break down the Aß peptides compared to the untreated peptides (PBS), Obviously, many more experiments must be performed and additional controls will have to be run before we can conclusively state that the antibodies are catalytically hydrolyzing the Aß peptide at the right site. Various naturally occurring proteases will be tested in this system so that we can identify the cleavage site of the antibodies by comparison with the known specificity of the different enzymes. We will also sequence the cleaved Aß peptides.

i. Hybridoma production II: Another distinct hybridoma fusion was performed using the spleen of a mouse immunized with a KLH conjugate of the statine (**Sta**) transition state analogs encompassing the carboxy-terminal region of Aß (Fig. 8) (Cys-Met-Val-Gly-Gly-Val/**Sta**-Val/**Sta**-Ile/**Sta**-Ala-Thr). ELISA was used to demonstrate antibody binding to both the normal $Aß_{1-43}$ peptide and to the statine transition state Aß peptide (Fig. 13).

The antibodies bound to the C-terminal locus on these carrier-free Aß peptides adsorbed directly to the microtitre plate, confirming their anti-peptide specificity. Most of the antibodies preferentially recognized the statine Aß transition state but cross-reacted with native $Aß_{1-43}$. This suggests that these new antibodies may be able to force the native Aß peptide into a conformation resembling the transition state for hydrolytic cleavage of its C-terminal amino acids. Such cleavage would in effect convert $Aß_{1-43}$ into potentially less harmful shorter peptides, like $Aß_{1-40}$ or $Aß_{1-39}$.

Clone 11E9 had the strongest binding preference for the statine analog and might therefore be the best prospect for having catalytic activity (Fig. 13). Several clones displayed no difference in their reactivity with the native versus statine transition state Aß peptide. We also tested the clones with $Aß_{1-40}$ to identify antibodies which do not react with this shortened, 40 amino acid version of Aß (Fig. 13). Used therapeutically, such antibodies would preferentially bind/cleave the less abundant but more noxious $Aß_{1-43}$ species in the blood as opposed to the smaller and less detrimental $Aß_{1-40}$.

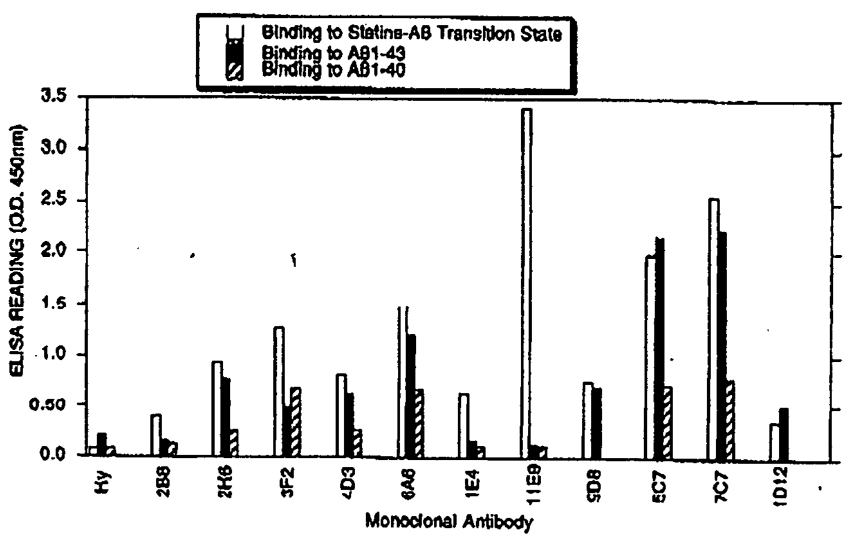


Fig. 13 ELISA Comparing Antibody Binding to Transition State Versus Native Aß

j. Disaggregation of β-Amyloid by Monoclonal Antibodies: The self-aggregation of synthetic Aβ peptides leads to microscopic structures which resemble amyloid plaques in the brain (16, 17) and exhibit the same bright green fluorescence upon exposure to thioflavin T (Fig. 14). These aggregates are very stable and usually require harsh detergents or strong acids to dissolve. However, it has now been demonstrated that the binding of certain anti-Aβ monoclonal antibodies can effectively inhibit the initial aggregation of this peptide and also disaggregate preformed Aβ complexes (16, 17).

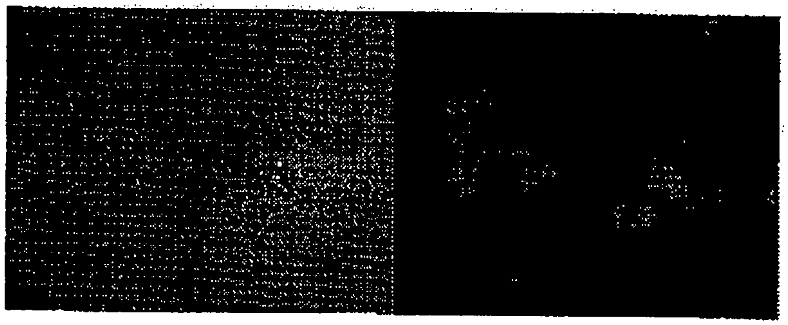


Fig. 14 Photomicrographs of Self-Aggregated Aβ Peptide

We set up a radioactive assay as a quick way to screen the different monoclonal antibodies produced in this laboratory for an ability to dissolve preformed Aβ aggregates. After adding ^{125}I-Aβ to unlabeled soluble peptide, aggregates were formed by bringing the solution to pH 5 or by stirring it overnight in PBS. An aliquot of the labeled aggregate was incubated for 1hr with either PBS, the 5A11 anti-Aβ antibody or an equal amount of an irrelevant mouse antibody (7D3, anti-human transferrin receptor). After centrifugation, the level of radioactivity in the precipitate was measured (Table II). The fact that the Aβ-specific 5A11 antibody solubilized 80% of the Aβ aggregates while an equal amount of the control antibody had only a minor effect suggests that the equilibrium was displaced by antibody-mediated binding of soluble Aβ.

Table II Solubilization of ^{125}I-Aβ$_{1\text{-}40}$ Aggregate by Monoclonal Anti-Aβ Antibody

Addition	^{125}I-Aβ$_{1\text{-}40}$ in Ppt. (cpm)	Amount Solubilized (% of PBS Control)
PBS control	3,420	-
+ 5A11 anti-Aβ	676	80
+ 7D3 anti-TfR	2,458	27

C.2 Produce Vectorized Anti-Aβ/Anti-Receptor Bispecific Antibodies

a. Vectors for transcytosis across the blood-brain barrier: Anti-transferrin receptor antibodies (anti-TfR) are the primary vectors that we will deploy for delivery of the anti-Aβ antibodies into the brain. The 7D3 mouse monoclonal antibody developed in this lab, is specific for the human receptor and selectively immunostains cortical capillaries in normal human brain tissue (48) (Fig. 15).

Fig. 15 Human Brain Capillaries Stained with the 7D3 Anti-TfR (48)

Antibody attachment to the receptor is not blocked by an excess of human transferrin. The epitope recognized by this antibody is therefore distant from the receptor-ligand binding site. Bispecific antibodies constructed with this 7D3 antibody and an anti-Aß antibody would be potentially useful for therapy in patients with Alzheimer's disease and possibly for preclinical trials in primates.

For studies in the transgenic mouse model of Alzheimer's disease we have obtained an anti-mouse transferrin receptor monoclonal antibody produced in the rat. This antibody also appears to recognize a transferrin receptor epitope which does not involve ligand binding. The antibody therefore has no effect on cell proliferation when tested using murine lines.

b. Synthesis of bispecific antibodies: The anti-Aß antibodies have been chemically coupled to anti-human transferrin receptor and anti-mouse transferrin receptor antibodies by different methods (26, 30). We adopted a rapid thioether linkage technique to form strictly bispecific hybrids using Traut's reagent and the heterobifunctional SMDP reagent. One component was sparingly substituted with thiol groups (SH). These readily reacted to form a thioether linkage upon mixture with the maleimido-substituted (M) second component.

$$Ab_A\text{-SH} + Ab_B\text{-M} \rightarrow Ab_A\text{-S-}Ab_B$$

Gel filtration of the reaction mixture on an S-300 column yields the purified dimer which is 300kDa and has two sites for binding Aß plus two sites for attachment to transferrin receptors on brain capillary endothelial cells. $F(ab')_2$ fragments of the two different antibody types will be similarly thioether-linked to form Fc-devoid reagents that cannot bind complement which might otherwise cause neurotoxic effects.

Non-targeted control hybrids have been formed by linking a nonspecific MOPC antibody to the anti-Aß antibody. This hybrid antibody will bind Aß, but, being non-reactive with transferrin receptors, should not cross the blood-brain barrier.

Smaller bispecific hybrids (100kDa) have been formed by reducing the intrinsic disulfides which link the heavy chains of $F(ab')_2$ fragments (49). The thiols generated were stabilized and Ellman's reagent (E) was used to activate these groups on one of the components (50). Exclusively bispecific $F(ab')_2$ hybrids were formed upon mixing the reduced Fab' with an activated Fab' having the alternate specificity.

$$Fab'_A\text{-SH} + Fab'_B\text{-SS-E} \rightarrow Fab'_A\text{-SS-}Fab'_B + \text{E-SH}$$

Purification on an S-200 column provided hybrids with one site for binding Aß and one site for interaction with the target epitope on the brain capillary endothelial cells.

A similar approach can be used to make even smaller disulfide linked single chain Fv heterobispecific dimers, Fv_A-SS-Fv_B (50kDa), to cross the blood-brain barrier. Soluble Fvs have been constructed to possess a carboxyl-terminal cysteine to facilitate the disulfide exchange shown below and create 50kDa heterodimers exclusively.

$$Fv_A\text{-SH} + Fv_B\text{-SS-E} \rightarrow Fv_A\text{-SS-}Fv_B + \text{E-SH}$$

In side by side comparisons between whole antibody and either Fab' or Fv based bispecific reagents, the latter have proven to be moderately more effective on a molar basis for cell uptake via the transferrin receptor-mediated pathway. Since these smaller constructs are monovalent for the cell-surface epitope, those findings dispel the notion that cross-linking of two surface receptors is necessary for the cellular uptake of immunocomplexes.

c. Characterization of bispecific antibodies: After the synthesis, purification and size analysis of the anti-Aß/anti-transferrin receptor bispecific antibody was completed, a series of functional assays were performed. Its ability to attach to transferrin receptor bearing human cells was confirmed by cytofluorimetry using an anti-mouse IgG probe (Fig. 16). The capacity of the hybrid reagent to bind ^{125}I-Aß compared favorably with that of the parent anti-Aß antibody (Table III).

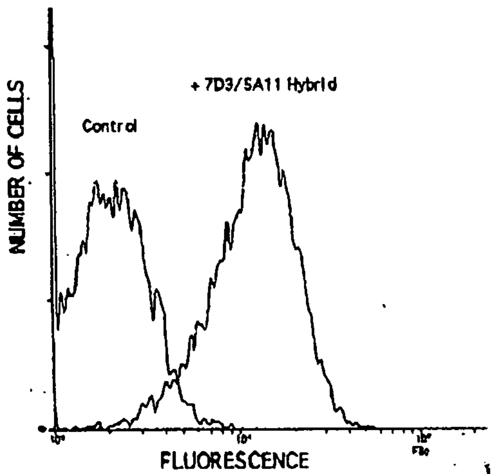


Fig. 16 Attachment of Bispecific Antibody to Receptor-Positive Cells

Table III ^{125}I-Aß Binding to Bispecific Antibody

Addition	^{125}I-Aß$_{1-40}$ Bound (cpm)
Control	4,199
+ anti-Aß	23,301
+ anti-Aß/anti-receptor	23,850

To ensure that both of these binding activities resided on the bispecific antibody we treated transferrin receptor positive cells with the hybrid reagent, washed away unbound material and then exposed these cells to ^{125}I-Aß$_{1-40}$. The cells were washed and the amount of cell-bound radioactivity was compared to control cells which had been identically prepared except that pretreatment with bispecific antibody was omitted.

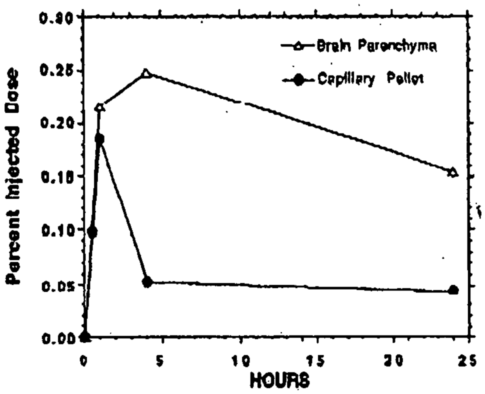
Table IV Bispecific Antibody-Mediated Binding of ^{125}I-Aß to Receptor-Positive Cells

Pretreatment of Cells	^{125}I-Aß$_{1-40}$ Bound (cpm)
None	2,367
+ anti-Aß/anti-transferrin receptor	11,476

The results (Table IV) verify the dual specificity of this bispecific antibody by clearly showing that it can simultaneously attach to the cell membrane and bind ^{125}I-Aß$_{1-40}$.

The vectorized bispecific antibody prepared for use in the transgenic mouse model of Alzheimer's disease is composed of a rat monoclonal antibody directed against the mouse transferrin receptor plus the 5A11 mouse anti-Aß monoclonal antibody. Both components were detected on the cell membrane by cytofluorimetry (Fig. 16) when this duplex was reacted with transferrin receptor positive mouse cells and probed using either a rat IgG-specific or mouse IgG-specific fluorescent secondary antibody reagent (data not shown).

d. Transcytosis of bispecific antibody into the brain: We coupled a rat monoclonal anti-mouse transferrin receptor antibody to a mouse monoclonal antibody so that the entry of this new vectorized bispecific construct into brain could be monitored. The bispecific antibody was labeled with ^{125}I and injected i.v. into normal mice. After different times the mice were sacrificed and the amount of ^{125}I-bispecific antibody which crossed the blood-brain barrier and entered the brain was determined by a mouse capillary depletion method (25, 51).



The amount of vectorized bispecific antibody found in the brain parenchyma or brain capillary fractions was measured following differential density centrifugation of the brain homogenate. These values were plotted as a function of time after i.v. injection (Fig. 17). The time-dependent redistribution of radiolabeled bispecific antibody from the capillaries and into the parenchyma is consistent with its passage across the cerebral endothelial blood-brain barrier (20). These preliminary experiments, which were performed in normal mice, will be repeated in plaque-bearing transgenic mice, when they reach 1-year of age. Greater accumulation in the parenchyma may result if the antibodies attach to Aß in the cerebral plaques.

Fig. 17 Transcytosis of the Vectorized Bispecific Antibody into Brain

e. Monitoring the brain distribution of bispecific antibody in live mice: An ability to follow the entry and accumulation of vectorized bispecific antibodies in the brain of live mice would assist in developing the intracerebral treatment of plaque-bearing mice. Time-course studies could be easily carried out and problems with inter-mouse variability would be greatly reduced. We therefore have begun preliminary studies with ^{125}I-labeled bispecific antibodies, to determine if immunoscintigraphy is feasible in this system. As a first step, we administered either the radiolabeled vectorized bispecific antibody (^{125}I-R17/5A11) or a non-vectorized control bispecific antibody to separate mice. Sequential brain images were accumulated at 1, 6, 24 and 48 hours following i.v. administration of the ^{125}I-labeled bispecific antibody probes. This technique suffers because it is difficult to determine how much of the signal is due to the levels of blood-borne radioactivity circulating through the brain. However distinctions were noted in the brain of mice treated with the mouse transferrin receptor reactive bispecific antibody versus those receiving the control bispecific antibody (Fig. 18, color images provided in the Appendix). When the vectorized agent was used, brain levels increased between 1 and 6 hrs and then declined to a much lower level at 24 and 48hrs. Mice treated with the control displayed no increase between 1 and 6 hrs. The reason for decreased brain levels at 24 hrs and beyond is not known but this might be due to dehalogenation of the bispecific antibody probes so that free ^{125}I is released and exits the brain. Alternative radioactive labels such as ^{111}In (52) or ^{99m}Tc (53) will be attached to the vectorized bispecific antibody if the use of iodine presents a technical problem.

Fig. 18 Dorsal Aspect Brain Images of Mice (Nose to Right) Treated with Vectorized (bottom) or Control (top) Bispecific Antibody; Darker Central Region in the Image Denotes Greater CPM

Further studies and additional controls will be run to determine the significance of the apparent differential distribution of these ^{125}I-labeled probes. For example we will co-inject a large excess of unlabeled vector antibody (anti-mouse TfR) with the ^{125}I-labeled vectorized bispecific antibody in an attempt to block its receptor-mediated entry into the brain. This imaging technology will be useful for determining if smaller vectorized bispecific antibodies (eg. $F(ab')_2$) with different physical properties and an altered biodistribution will penetrate into the brain more effectively. Importantly, the digtital scintigraphy data shown above can be easily quantified using standards and the integration functions provided in the analysis software. For example, the central dark region in the brain image shown above corresponds to $\sim 1.5 \times 10^5$ cpm.

C.3 Disruption of Plaque Development in Transgenic Mice

a. The Tg2576 transgenic mouse colony: Transgene-positive Tg2576 mice for breeding were generously provided by Dr. Karen Hsiao (44). She provided us with the pertinent breeding information concerning these mice and has offered to answer questions if any problems arise. Our animal care technicians are well experienced in handling transgenic mice and have established a healthy colony.

Dr. Hsiao recommended that we perform genotyping on the Tg2576 mice both before and after each experiment. The 3'UT of the hamster cosmid PrP vector will be used as a hybridization probe (45) and we have received a sample of this region. Ear punch biopsy DNA has been prepared and PCR procedures have been carried out on every weaned mouse to identify mice bearing the transgene (45) (data not shown). We now routinely process and cut brain sections for immunocytochemical and thioflavin S detection of amyloid plaques in these mice (Fig. 19). Aß in brain tissue will also be extracted and quantified by ELISA as previously described (45).

We currently have a large number of young transgene positive mice which are available for use to perform this ongoing research. Many one year-old experimental animals with preestablished amyloid plaques (Fig. 19) are also now ready for our proposed experimental studies on the cerebral delivery of anti-Aß antibodies.

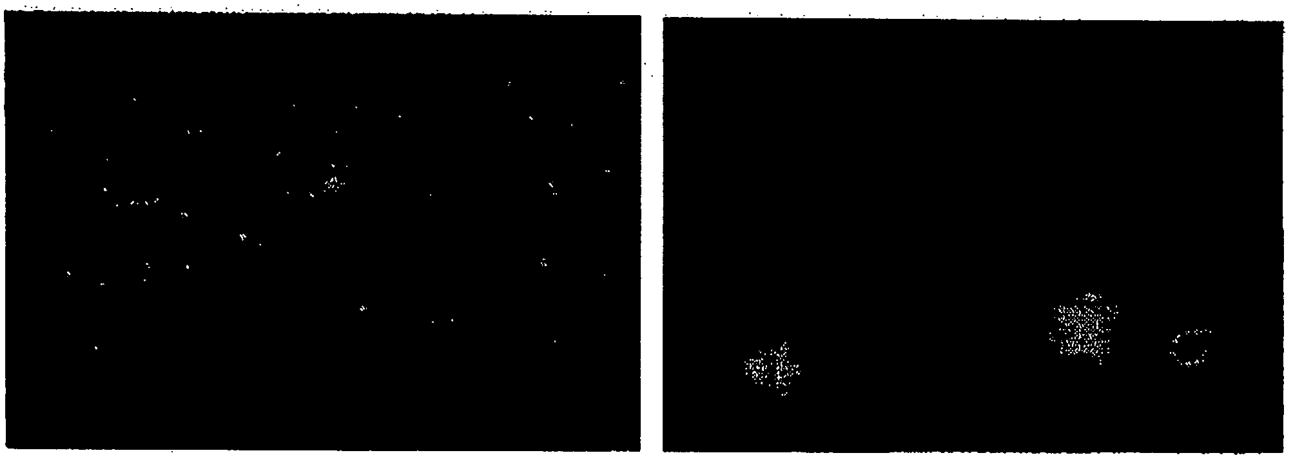


Fig. 19 *Low- and High-Power Micrograph of Thioflavin Stained Brain Plaques from Our Tg Positive Mice*

Farrell & Associates, P.C.
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(207) 363-0558

May 31, 1999
Client No. BBRI-2004M

Boston Biomedical Research Institute
P.O. Box 981
Douglas MA 01516

Attn: Ms. Pamela Torpey

B-Amyloid Peptide

Previous Balance $8,499.50

Fees

	Rate	Hours	
05/04/99 SYH Studying comments from inventor (VR) re: the Exemplification section; Incorporating inventor comments into Application.	100.00	2.70	270.00
05/12/99 SYH Incorporating recently received comments from inventor (VR) into Exemplification; Preparing most recent draft of the Application for transmission to Pamela Torpey.	100.00	0.70	70.00
05/20/99 SYH Conferring with inventor (VR, SYH) re: newly provided information; incorporating new information into Application.	100.00	2.00	200.00
05/21/99 SYH Editing Application.	100.00	4.80	480.00
05/25/99 SYH Drafting Summary and Abstract of the Invention; transmitting draft Application to inventor (VR) for review prior to filing	100.00	2.00	200.00
For Current Services Rendered		12.20	1,220.00

Expenses

05/13/99	Postage expense	5.50
05/13/99	Photocopy charges	33.75

Boston Biomedical Research Institute

May 31, 1999
Client No. BBRI-2004M

B-Amyloid Peptide

05/25/99	Postage expense	2.97
05/25/99	Photocopy charges	3.50

	Total Expenses	45.72
	Total Current Work	1,265.72
	Balance Due	$9,765.22
		=========

Aged Due Amounts

0-30	31-60	61-90	91-120	121-180	181+
1,265.72	60.00	7,403.50	1,036.00	0.00	0.00

Please Remit	$9,765.22
	=========

EXHIBIT A

FARRELL & ASSOCIATES, P.C.

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Kevin M. Farrell

Shayne Y. Huff, Ph.D.
Technical Specialist*

Richard L. Sampson
Of Counsel

May 13, 1999

Ms. Pamela Torpey
P.O. Box 981
Douglas, MA 01516

Re: BBRI-2004
Immunological Control of Beta-Amyloid Levels In Vivo

Dear Pamela:

Enclosed please find a draft patent application, prepared by Dr. Shayne Huff, based on the above-referenced invention disclosure. The application is in near-final form, but we have requested characterization of antibodies produced against molecules which mimic the transition states of the beta-amyloid peptide. We are claiming such antibodies, we understand that such antibodies have been produced, and we feel that from an enablement standpoint, this support would be very helpful. If gathering this information is unduly burdensome at this time we can, of course, file without it.

I have made a note to contact you next week regarding this case. We do not want to hold up the filing if the data referred to above will not be available in the very near term. Any comments from Dr. Raso can be incorporated in short order.

Best regards,

Kevin

Kevin M. Farrell

KMF:tlw

cc: Victor Raso, Ph.D., w/enc.
Shayne Y. Huff, Ph.D., w/o enc.

BBRI\ARCH\2004.L1

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Kevin M. Farrell

Shayne Y. Huff, Ph.D.
Technical Specialist*

Richard L. Sampson
Of Counsel

May 25, 1999

Dr. Victor Raso, Ph.D.
Boston Biomedical Research Institute
20 Staniford St.
Boston, MA 02114

Re: BBRI-2004
Immunological Control of Beta-Amyloid Levels In Vivo

Dear Dr. Raso:

Enclosed please find a draft patent application, for your review. This revision differs from the draft transmitted to you on May 13, 1999. Please review the draft for accuracy and completeness. Note that there are a few questions addressed to you within the body of the text. We will incorporate any revisions indicated and file the application in short order (within hours of receipt of your comments).

Best regards,

Shayne Huff

Shayne Huff

cc: Ms. Pamela Torpey, w/enc.
Kevin Farrell, Esqire, w/o enc.

BBRI\CURR\2004.L1

DRAFT

IMMUNOLOGICAL CONTROL OF BETA-AMYLOID LEVELS IN VIVO

Background of the Invention

Alzheimer's disease is a progressive and ultimately fatal form of dementia that affects a substantial portion of the elderly population. Definitive diagnosis at autopsy relies on the presence of neuropathological brain lesions marked by a high density of senile plaques. These extracellular deposits are found in the neo-cortex, hippocampus and amygdala as well as in the walls of the meningeal and cerebral blood vessels. The principal component of these plaques, is a 39 to 43 residue β-amyloid peptide. Each plaque contains approximately 20 fmole (80 picograms) of this 4 kDa peptide (Selkoe et al., *J. of Neurochemistry 46*: 1820 (1986)). Apolipoprotein E and neurofibrillary tangles formed by the microtubule-associated tau protein are also often associated with Alzheimer's disease.

β-amyloid is proteolytically cleaved from an integral membrane protein called the β-amyloid precursor protein. The gene which codes for this protein in humans is found on chromosome 21 (St George-Hyslop et al., *Science 235*: 885 (1987), Kang et al., *Nature* 325: 733 (1987)). Numerous cultured cells and tissues (eg. brain, heart, spleen, kidney and muscle) express this β-amyloid precursor protein and also secrete the 4 kDa β-amyloid fragment into culture media, apparently as part of a normal processing pathway.

While it is difficult to establish an absolute causal relationship between β-amyloid or the plaques it forms and Alzheimer's disease, there is ample evidence to support the pathogenic role of β-amyloid. For example, patients with Down's syndrome have an extra copy of the β-amyloid precursor protein gene due to trisomy of chromosome 21 (St George-Hyslop et al., *Science 235*: 885 (1987), Kang et al., *Nature* 325: 733 (1987)). They correspondingly develop an early-onset Alzheimer's disease neuropathology at 30-40 years of age. Moreover, early-onset familial Alzheimer's disease can result from mutations in the β-amyloid precursor protein gene which fall within or adjacent to the β-amyloid sequence (Hardy, J., *Nature Genetics 1*: 233 (1992)). These observations are consistent with the notion that

deposition of β-amyloid as plaques in the brain are accelerated by an elevation in its extracellular concentration (Scheuner et al., *Nature Med. 2:* 864 (1996)). The finding that β-amyloid is directly neurotoxic both *in vitro* and *in vivo* (Kowall et al. *Proc. Natl. Acad. Sci. 88*: 7247 (1991)), ~~opens the possibility~~ suggest that soluble aggregated β-amyloid, not the plaques per se, may produce the pathology.

Observations have indicated that amyloid plaque formation may proceed by a crystallization type mechanism (Jarrett et al., *Cell 73*: 1055 (1993)). According to this model, the seed that initiates plaque nucleation is an β-amyloid which is 42 or 43 amino acids long ($A\beta_{1-43}$). The rate-determining nucleus formed by $A\beta_{1-43}$ or $A\beta_{1-42}$ allows peptides $A\beta_{1-40}$ or shorter to contribute to the rapid growth of an amyloid deposit. This nucleation phenomenon was demonstrated *in vitro* by the ability of $A\beta_{1-42}$ to cause the instantaneous aggregation of a kinetically stable, supersaturated solution of $A\beta_{1-40}$. That finding has led to the possibility that $A\beta_{1-40}$ might be relatively harmless in the absence of the nucleation peptides $A\beta_{1-42}$ or $A\beta_{1-43}$. Indeed, elevated levels of these long peptides have been found in the blood of patients with familial Alzheimer's disease (Scheuner et al., *Nature Med. 2:* 864 (1996)). Moreover, $A\beta_{1-42}$ or $A\beta_{1-43}$ was found to be the predominant form deposited in the brain plaques of many Alzheimer's disease patients (Gravina et al., *J. of Biol. Chem. 270*: 7013 (1995)).

Given the central role played by β-amyloid, it has become increasingly important to understand the interrelationship between the different pools of these molecules in the body. Free β-amyloid present in the blood most likely arises from peptide released by proteolytic cleavage of β-amyloid precursor protein present on cells in the peripheral tissues. Likewise most of the free β-amyloid found in the brain and cerebrospinal fluid is probably derived from peptide released by secretase cleavage of β-amyloid precursor protein expressed on brain cells. The

peptides are identical regardless of origin, and the results from several studies suggest an intercommunication between these pools.

Brief Description of the Figures

Figure 1 is an amino acid sequence listing (SEQ ID NO: 1) of the 43 residue β-amyloid peptide (Aβ).

Figure 2 is an amino acid sequence listing (SEQ ID NO: 2) of the antigenic peptide made from the N-terminal sequence of ~~Aβ~~ β-amyloid ($A\beta_{1-16}$).

Figure 3 is an amino acid sequence listing (SEQ ID NO: 3) of the antigenic peptide made from the central region of ~~Aβ~~ β-amyloid ($A\beta_{10-25}$).

Figure 4 is an amino acid sequence listing (SEQ ID NO: 4) ($A\beta_{35-43}$) of the antigenic peptide made from the C-terminal sequence of ~~Aβ~~. β-amyloid

Figure 5 is a diagrammatic representation of data from an ELISA comparing monoclonal antibody binding to $A\beta_{35-43}$ and $A\beta_{1-43}$ versus $A\beta_{1-40}$.

Figure 6 indicates the amide linkages in the peptide made from the ~~Aβ~~ β-amyloid C-terminal sequence (SEQ ID NO: 4) that were independently replaced with a statyl moiety, to generate the different statine transition state analogs of the peptide.

Figure 7 indicates the amide linkages in the peptide made from the ~~Aβ~~ β-amyloid central sequence (SEQ ID NO: 3) that were independently replaced with a statyl moiety, to generate the different phenylalanine statine transition state analogs of the peptide.

Figure 8 is a structural comparison between the native ~~Aβ~~ β-amyloid peptide and the transition state phenylalanine statine ~~Aβ~~ β-amyloid peptide analog.

Figure 9 is a structural comparison between the native ~~Aβ~~ β-amyloid peptide and the reduced peptide bond transition state ~~Aβ~~ β-amyloid peptide analog.

Figure 10 is a formulaic representation of the native C-terminal region of phosphonamidate transition state analog of β-amyloid, and the

the C-terminal region of ~~Aβ~~ β-amyloid ($A\beta_{35-43}$).

Figure 11 indicates the putative transition state for peptide hydrolysis by zinc peptidases, ~~and~~ compared to the phosphonate and phosphonamidate mimics.

Figure 12 is a structural comparison of the native ~~Aβ~~ β-amyloid peptide and the transition state phosphonamidate ~~Aβ~~ β-amyloid peptide which has the peptide link between Gly 38 and Val 39 replaced with a phosphonamidate bond.

Figure 13 is a diagrammatic representation of data from an ELISA which assess the binding of monoclonal antibodies, generated to transition state ~~Aβ~~ β-amyloid peptide analogs, to the normal $A\beta_{1-43}$ and to the phenylalanine statine transition state ~~Aβ~~ β-amyloid peptide.

Figure 14 is a diagrammatic representation of data from an ELISA, comparing antibody binding to the statine transition state ~~Aβ~~ β-amyloid peptide versus native $A\beta_{1-43}$. and native $A\beta_{1-40}$

Figure 15 is a graph of data showing the cleavage of 125I-Aβ-sepharose by monoclonal antibodies generated to transition state analogs of ~~Aβ~~ β-amyloid.

Figure 16 is a diagrammatic representation of data which ~~indicates~~ quantitates the attachment of bispecific antibody to receptor-positive cells.

Figure 17 is a diagrammatic representation of data ~~obtained~~ obtained from experiments following ~~indicating~~ the transcytosis of vectorized bispecific antibody into brain. designed to track

Detailed Description of the Invention

The present invention relates to immunologically based methods for controlling levels of β-amyloid in the body of an animal. The invention is based on the finding that antibodies specific for β-amyloid are able to bind β-amyloid in the presence of a physiological level of human serum albumin. The invention is also based on the finding that an animal can tolerate the presence of antibodies specific for β-amyloid in amounts sufficient to sequester β-amyloid in the bloodstream.

One aspect of the present invention related to a method for sequestering free β-amyloid in the bloodstream of an animal. The soluble and insoluble forms of β-amyloid present within an animal are in dynamic equilibrium. Soluble β-amyloid is thought to translocate between blood and cerebrospinal fluid. Insoluble β-amyloid aggregates deposit from the soluble pool in the brain, as amyloid plaques. Results detailed in the Exemplification section below indicate that intravenous administration of antibodies specific for β-amyloid to an animal impedes the passage of soluble β-amyloid out of the peripheral circulation. This occurs because the β-amyloid specific antibodies, which are restricted to the peripheral circulation, bind to β-amyloid and sequester it in the circulation. Such sequestration is accomplished through intravenous administration of an appropriate amount of antibodies specific for β-amyloid to the animal. The amount of antibody ~~administered~~ which is sufficient to produce sequestration when administered is dependent upon various factors (e.g. specific characteristics of the antibody to be delivered, the size, metabolism, and overall health of the animal) and ~~should~~ are to be determined on a case by case basis.

Administered antibodies can be monoclonal antibodies, a mixture of different monoclonal antibodies, polyclonal antibodies, or any combination therein. In one embodiment, the antibodies bind to the C-terminal region of β-amyloid. Such antibodies specifically bind the less abundant, but more noxious $A\beta_{1-43}$ species in the blood as opposed to the smaller and less detrimental $A\beta_{1-40}$. In another embodiment, a combination of antibodies having specificity for various regions of β-amyloid are administered. In another embodiment, antibodies which catalyze the hydrolysis of β-amyloid, discussed in more detail below, are administered either alone or in combination with other anti-β-amyloid antibodies.

The animal to which ~~is administered~~ the antibodies are administered is any animal which has circulating soluble β-amyloid. In one embodiement, the animal is a human. The human

may be a healthy individual, or alternatively, may be suffering from or at risk for a disease in which elevated β-amyloid levels are thought to play a role, for example a neurodegenerative disease such as Alzheimer's disease.

A related aspect of the present invention is a method for sequestering free β-amyloid in the bloodstream of an animal by stimulating ~~generating~~ an immune resonse ~~in~~ within the animal ~~to~~ endogenous β-amyloid. The results detailed in the Exemplification below indicate that an animal can tolerate the induction of an immune response which produces antibodies to endogenous β-amyloid, and that the presence of such antibodies will alter the distribution of β-amyloid in the body, in a similar manner as the above described method of administering β-amyloid binding antibodies. The immune response to endogenous β-amyloid is generated by immunizing the animal with one or more antigens comprised of epitopes present on the endogenous β-amyloid ~~endogenous to the animal~~. Epitopes present on the innoculated antigens can correspond to epitopes present on any region of the β-amyloid molecule. In a preferred embodiment, epitopes found on the C-terminal region of β-amyloid are used to generate antibodies which specifically bind the $A\beta_{1-43}$ species as opposed to the smaller $A\beta_{1-40}$. In an alternate embodiment, a combination of different epitopes ~~and antigen containing epitopes~~ are administered to generate a variety of antibodies to β-amyloid. A more generalized immune response is generated by immunizing either with a mixture of different small peptide antigens or with the full-length 43 residue β-amyloid peptide. In another embodiment, antigens used for inoculation include transition state analogs of β-amyloid peptides to induce antibodies which have catalytic activity directed towards β-amyloid hydrolysis, described in detail below.

The immunoreactivity of the antigens can be enhanced by a variety of methods, many of which involve coupling the antigen to an immunogenic carrier. In addition, various methods are known and available to one of skill in the art for specifically

enhancing the immunogenicity of endogenous molecules or the epitopes contained therein. Various ~~M~~modifications can be made to the β-amyloid antigen(s) described herein to render it more compatible for human use. For example, the peptide(s), can be genetically engineered into appropriate antigenic carriers, or DNA vaccines can be designed.

The above techniques for sequestering β-amyloid in the circulation are also useful for reducing ~~brain β-amyloid levels~~ the levels of β-amyloid in the brains. Because the formation of amyloid plaques in the brain is dependent, at least in part, on the levels of free β-amyloid present in the brain, reducing brain β-amyloid levels of an animal will in turn reduce the formation of amyloid plaques in the brain. Therefore, the above techniques are ~~also~~ useful for preventing the formation of amyloid plaques in the brain of an animal. This is especially applicable to an animal which is considered at risk for the development of amyloid plaques; a risk which may result from a genetic predisposition or from environmental factors. Administration of antibodies, or immunization of the animal to produce endogenous antibodies, ~~for~~ to β-amyloid can be of therapeutic benefit to such an animal (e.g. a human who has a family history of Alzheimer's disease, or who is diagnosed with the disease).

Another aspect of the present invention relates to antibodies which are characterized by the ability to catalyze the hydrolysis of β-amyloid at a predetermined amide linkage. Experiments detailed in the Exemplification section demonstrate the generation of different antibodies which have proteolytic activity towards β-amyloid. Such antibodies are generated by immunizing an animal with a~~n~~ antigen which is a ~~β-amyloid peptide~~ antigen which is a ~~n analog~~ statis transition state (oxoy) ~~analog~~ of the B-amyloid peptide. A transition state analog mimics the ~~transition~~ of the hydrolysis state that β-amyloid adopts during hydrolysis ~~at~~ of a specific ~~predetermined~~ amide linkage. Transition state analogs useful for generating the catalytic antibodies include, without limitation, statine, phenylalanine statine, phosphonate, phosphonamidate, and

of an amide linkage within the B-amyloid peptide.

reduced peptide bond transition state analogs.

Antibodies generated to epitopes unique to the transition state preferentially bind β-amyloid in the transition state. Binding of these antibodies stabilizes the transition state, which leads to hydrolysis of the corresponding amide bond. The particular amide linkage to be hydrolyzed is chosen based upon the desired cleavage product. For example, cleavage of full length β-amyloid into two peptide fragments which cannot aggregate into amyloid plaques would be of therapeutic use in the methods disclosed herein. Monoclonal antibodies which recognize the transition state of specific amide likages in β-amyloid include ..... **[VR: PLEASE PROVIDE LIST OF AMIDE LINKAGES THAT THE MONOCLONAL ANTIBODIES RECOGNIZE.]**

At least two different classes of antibodies are generated by the above methods. The first class preferentially binds the transition state analog, and also detectably cross reacts with natural β-amyloid when using ~~the~~ an ELISA ~~detailed in~~ as the Exemplification ~~section~~, to detect binding. The second class binds the transition state analog, and does not ~~detectably~~ cross react to natural β-amyloid at levels which are detectable via ~~using the~~ ELISA (see the procedure detailed in the Exemplification section ~~to detect binding~~ for specific ELISA procedures.). Both classes of antibodies have potential value as catalytic antibodies. ~~The~~ These ~~respective~~ binding characteristics ~~affinities of an anti-transition state antibody~~ characteristics of native vs. transition state ~~is~~ are likely to reflect ~~its~~ the ~~activity at catalyzing hydrolysis~~ catalysis activity of the antibody. It is thought that in order for an antibody to ~~have activity at~~ ~~catlyzing~~ catalyze hydrolysis of a protein, it must posess at least a minimal ability to bind the natural (non-transition) sp? state of the protein. Antibodies which retain significant binding for β-amyloid, (that e.g. antibodies strongly cross react with natural β-amyloid) may be more efficient at catalyzing hydrolysis due to a higher efficiency of binding the β-amyloid. Once bound, these antibodies force the protein into a transition state conformation for hydrolytic cleavage. Alternatively, antibodies which only minimally cross react with natural β-amyloid, although less

efficient at binding native β-amyloid, are likely to be more efficient at forcing the bound β-amyloid into the transition state conformation for hydrolytic cleavage. It should be pointed out that failure to detect binding of the anti-transition state antibodies to natural β-amyloid by the ELISA methods presented in the Exemplification herein does not necessarily reflect an inability to bind natural β-amyloid sufficiently to function as a catalytic antibody. More likely, a lack of detection merely reflects the sensitivity limitations of the assay.

~~Note~~ Antibodies ~~with~~ which have substantial affinity for the predicted cleavage products of the native β-amyloid peptide may be subject to product inhibition and might therefore exhibit low turnover. Such undesireable antibodies can be identified by secondary screening using peptides which contain epitopes of the predicted cleavage products (e.g. via ELISA).

In a preferred embodiment, the antibodies are monoclonal. monoclonal antibodies ~~These can be~~ are produced by immunizing an animal (e.g. mouse, guinea pig, or rat) with the transition state analog antigen, and subsequently producing hybridomas from the animal, by standard procedures. Hybridomas which ~~producing~~ produce the desired monoclonal antibodies are then identified by screening. One example of a screening method is presented in the Exemplification section which follows. In another embodiment, the antibodies are polyclonal. Polyclonal antibodies are generated by immunizing an animal (e.g. a rabbit, chicken, or goat) with antigen and obtaining sera from the animal. Polyclonal antibodies ~~with~~ which have the desired binding specificities can be further purified from the sera by one of skill in the art through the course of routine experimentation.

Another aspect of the present invention is the use of statine and reduced peptide bond analogs to elicit catalytic antibodies having proteolytic activity. The Exemplification section below details methods for using statine analogs as antigen in the production of catalytic antibodies, and also lists examples of anti-transition-state antibodies generated using these methods. The "statyl" moiety is derived from naturally

evolved protease transition state inhibitors like amastatin, pepstatin, and bestatin. These naturally-occurring statine-based inhibitors have been used to effectively block the activity of aminopeptidases, aspartic proteases and the HIV protease. Synthetic peptides containing a statine residue offer novel features for the induction of catalytic antibodies. The statyl moiety has a tetrahedral bond geometry, its length is extended by two CH_2 units, it has a strategically placed OH group and the structure has no charge. The presence of the additional CH_2 units is expected to elicit a more elongated antibody combining site, and antibodies generated to this site will induce extra strain on the peptide substrate, producing an accellerated catalysis. In addition, the OH group in these statine analogs is thought to better approximate the position and chemistry of the true transition state. Statine-based transition-state analogs should therefore elicit a class of antibodies which is significantly different from those obtained from the more commonly used negatively charged phosphonate analogs.

Reduced peptide bond analogs introduce a tetrahedryl configuration, without increasing the distance between amino acid residues. This feature should more closely approximate the true transition state geometry, than previously used analogs. A positively charged secondary amine replaces the amide nitrogen of the natural polypeptide and should elicit a complementary negatively charged side chain at a proximal locus in the antibody combining site. The presence of Such ancillary glutamyl or aspartyl groups ~~present~~ on the antibody ~~could~~ will assist antibody-mediated catalysis of peptide cleavage via acid-base exchange. Reduced peptide bond-based transition-state analogs should therefore elicit a class of antibodies which is significantly different from those obtained from using the more commonly used negatively charged phosphonate analogs. Both reduced peptide bond analogs and ~~also~~ statine analogs can be used to produce a wide variety of specific transition state analog antigens ~~which mimic a wide variety of~~ proteins ~~or~~ for a polypeptides. These antigens can in turn be used to generate the respective catalytic antibodies. ~~to a wide ran~~

Administration of the β-amyloid catalytic antibodies described above finds use in the above described methods for 1) sequestering free β-amyloid in the bloodstream of an animal, 2) reducing levels of β-amyloid in the brain of an animal, and 3) preventing the formation of amyloid plaques in the brain of an animal. Experiments presented in the Exemplification demonstrate that immunization of an animal with a transition state analog results in the generation of an immune response to produce antibodies which recognize the transition state, and which catalyze hydrolysis of the β-amyloid protein. This indicates that the transition state analogs can be used as antigens in these methods to induce the production of antibodies in the animal which recognize and catalyze cleavage of endogenous β-amyloid.

Methods which involve reducing overall levels of β-amyloid in an animal through the proteolytic action of the above described catalytic antibodies are also encompassed by the present invention. The presence of functional catalytic antibodies in the circulation of an animal reduces the level of β-amyloid in the circulation. Accordingly, the present invention provides a method for reducing levels of circulating β-amyloid in an animal by introducing the above described catalytic antibodies into the animal.

The present invention also provides a method for reducing levels of circulating β-amyloid in an animal by immunizing the animal with a β-amyloid transition state analog to induce antibody production. The use and design of such vaccines is described above, and examples are detailed in the Exemplification section below. Administration of the antibodies to the animal is preferably via intravenous administration. Such antibodies are either monoclonal, mixed monoclonal, polyclonal or any mixture thereof. The origin of the antibody may affect the half-life of the antibody in the animal; antibodies from less related species are more likely to be recognized as foreign by the animal's immune system. Preferably, administered antibodies are derived from a species closely related to the animal, to maximize half-

life and minimize adverse reactions by the host. Administration of isolated variable region antibody fragments may produce beneficial results in this regard.

The reduction of β-amyloid levels in the circulation of an animal is expected to displace the equilibrium of β-amyloid in the body, and lead to a reduction in the levels of β-amyloid in the brain of the animal through mass action. In this respect, the present invention provides methods for reducing the levels of β-amyloid in the brain of an animal, by either administering catalytic antibodies to the animal, or by administering a transition state analog to induce endogenous antibody production. It follows that these procedures also have value as methods for preventing the formation of amyloid plaques in the brain of an animal, since the resulting reduction in the levels of β-amyloid in the brain of an animal should prevent the formation of amyloid plaques. These procedures also have value as methods for disaggregating amyloid plaques present in the brain of an animal, since evidence indicates that lower brain β-amyloid levels can lead to the disaggregation of plaques.

Another aspect of the present invention ~~also~~ provides ~~for~~ a more direct method~~s~~ of altering the distribution of β-amyloid in the brain~~s~~ by actually delivering anti-β-amyloid antibodies to the brain. Methods described above for reducing levels of β-amyloid in the brain and for preventing aggregation of amyloid plaques depend upon exchange between β-amyloid pools in the circulation the cerebrospinal fluid, the exchange being driven by a disruption of the equilibrium between the pools. In contrast, delivery of anti-β-amyloid antibodies to the brain will directly affect β-amyloid aggregation. Evidence presented in the Exemplification section below indicates that the binding of certain anti-β-amyloid antibodies inhibits the initial aggregation of β-amyloid *in vitro*, and also disaggregates preformed *in vitro* β-amyloid complexes. Moreover, if insoluble peptide is in equilibrium with a low level of soluble β-amyloid, then an anti-β-amyloid binding

antibody could upset this balance and gradually dissolve the precipitate. These observations indicate that the presence of β-amyloid antibodies in the brain will directly inhibit the formation of amyloid plaques and will also disaggregate preformed plaques by disrupting the dynamic equilibrium between soluble β-amyloid and fibrillar β-amyloid deposited as plaques. Furthermore, a highly active catalytic antibody is expected to destroy insoluble β-amyloid plaques by hydrolytically cleaving the constituent aggregated peptides.

One way of delivering antibodies to the brain is by producing vectorized antibodies competent for transcytosis across the blood-brain barrier. Vectorized antibodies are produced by covalently linking an antibody to an agent which promotes delivery from the circulation to a predetermined destination in the body. Examples of such vectorized antibodies can be found in the prior art .... **[VR: PLEASE PROVIDE REFERENCES]** One such agent is another antibody which is directed towards a cell surface component, such as a receptor, which is transported away from the cell surface. Examples of antibodies which confer the ability to trancytose the blood-brain barrier include, without limitation, anti-insulin receptor antibodies, and also anti-transferrin receptors (Saito et al., *Proc Natl Acad Sci USA 92*: 10227-31 (1995); Pardridge et al., *The Primate 12*: 807-816 (1995); **[VR: PLEASE VERIFY THE ACCURACY OF THIS LAST REFERENCE AS IT WAS NOT CLEAR IN THE MATERIALS PROVIDED]** Broadwell et al., Exp Neurol 142: 47-65 (1996)). This first antibody is covalently linked to an antibody which binds β-amyloid. Alternatively, coupling the β-amyloid antibodies to ligands which bind these receptors, (e.g. insulin, transferrin, or LDL **[VR: PLEASE PROVIDE COMPLETE NAME OF LDL]**) will also produce a vectorized antibody competent for delivery to the brain from the circulation (Descamps et al., *Am. J. Physiol. 270*: H1149-H1158 (1996); Duffy et al., *Brain Res. 420*: 32-38 (1987); Dehouck et al., *J. Cell Biol. 138*: 877-889 (1997)).

A vector moiety can be chemically (or genetically) attached to

the anti-β-amyloid antibody to facilitate its delivery into the central nervous system. This vector component can be for example, an anti-transferrin receptor or anti-insulin receptor antibody ~~that~~ binds ~~to~~ th~~ose~~ receptors on the brain capillary endothelial cells (Bickel et al., *Proc Natl Acad Sci U S A* 90: 2618-22 (1993); Pardridge et al., *J Pharmacol Exp Ther 259*: 66-70 (1991); Saito et al. *Proc Natl Acad Sci U S A* 92: 10227-31(1995); Friden et al., *J. Pharm. Exper. Ther.* 278: 1491-1498 (1996)) which make up the blood-brain barrier. The ~~resulting~~ bifunctional antibody (Raso et al., *J. Biol. Chem.* 272: 27623-27628 (1997); Raso et al., *J. Biol. Chem.* 272: 27618-27622 (1997); Raso, V. *Anal. Biochem.* 222:297-304 (1994); Raso et al., *Cancer Res* 41: 2073-2078 (1981); Raso et al., Monoclonal antibodies as cell targeted carriers of covalently and non-covalently attached toxins. *In* Receptor mediated targeting of drugs, vol. 82. G. Gregoriadis, G. Post, J. Senior and A. Trouet, editors. NATO Advanced Studies Inst., New York. 119-138 (1984)) will attach to appropriate receptors on the luminal side of the vessel. Once bound to the receptor, both components of the bispecific antibody ~~can~~ pass across the blood-brain barrier by the process of transcytosis. Anti-β-amyloid antibodies which have entered the brain interact directly with both β-amyloid plaques and the soluble β-amyloid pool. It has ~~been~~ estimated that concentrations of macromolecules in the 10^{-8}-10^{-7} M range can be achieved in the brain using vector-mediated delivery via these brain capillary enriched protein target sites (Maness et al., *Life Sciences* 55: 1643-1650 (1994); Lerner et al., *Science* 252: 659-667 (1991)). Importantly, the vector appears safe since animals dosed daily for two weeks with an anti-transferrin receptor antibody ~~showed~~ no loss of integrity of the blood-brain barrier using a radioactive sucrose probe (Broadwell et al., *Exp Neurol 142:* 47-65 (1996)).

The Exemplification details the production of vectorized bispecific antibodies which bind β-amyloid. The bispecific

antibodies transcytose across the blood brain barrier via a first specificity which binds the transferrin receptor. Use of antibodies which bind the transferrin receptor for delivery of agents across the blood brain barrier is described by Friden et al. in U.S. Patents No. 5,182,107; No. 5,154,924; No. 5,833,988; and No. 5,527,527; the contents of which are incorporated herein by reference.

Results from experiments presented in the Exemplification section which follows indicate that the produced bispecific antibodies retain their separate specificities and are delivered across the blood-brain barrier into the brain parenchyma and brain capillaries of a live animal when administered intravenously.

Alterna~~tive~~l methods for the production of bispecific antibodies have been described for genetically engineering bispecific reagents or for producing them intracellularly by fusing the two different hybridoma clones **[VR: PLEASE PROVIDE REFERENCES FOR THESE METHODS]**. Vectorized bispecific antibodies produced by these techniques can also be used in the methods of the present invention.

Since ~~Because~~ the introduction of whole antibodies into the brain might be detrimental if they were to fix complement and promote complement-mediated lysis of neuronal cells, it may be beneficial to produce and utilize smaller vectorized $F(ab')_2$ bispecific reagents. ~~can be produced~~. It has been shown that aggregated β-amyloid itself can fix complement in the absence of any antibody and that the resulting inflammation may contribute to the pathology of Alzheimer's disease. The possibility of intracerebral antibody having a similar effect can be ~~would be~~ greatly reduced by eliminating the Fc region of the antibody. Moreover, since coupling of Fab' halves uses the intrinsic hinge region cysteines, no extraneous substituent linkage groups need ~~to~~ be added. Faster or more efficient entry into the brain represents another potential advantage that smaller $F(ab')_2$ or Fv_2 reagents may provide for intracerebral

delivery. In addition, the two types of vectorized molecules may have different biodistribution and plasma half-life characteristics (Spiegelberg et al., *J. Exp. Med.* 121: 323 (1965)).

7

re-write

Depending on their design, anti-β-amyloid bispecific antibodies ~~situated~~ in the brain potentially ~~can~~ function by ~~in three different ways to~~ mechanisms can reduce soluble β-amyloid and β-amyloid deposits by 3 potential mechanisms. ~~An~~

3 different mechanisms.

anti-β-amyloid bispecific antibody that tightly binds soluble β-amyloid will not only sequester the peptide but, due to efflux of vectorized molecules from the central nervous system (Kang et al., *J. Pharm. Exp. Ther.* 269: 344-350 (1994)), ~~potentially can~~ may also carry the bound ~~Aβ~~ β-amyloid out of the brain and release it into the blood stream. ~~This~~ Such a clearance mechanism would lead to a continuous cycling of β-amyloid out of the brain.

add catalytic Abs 7

To be effective the anti-β-amyloid sites of a bispecific antibody must be empty before passage out of the blood and into the brain. Therefore the concentration of bispecific antibody in animals must exceed the level of β-amyloid circulating in the blood. Calculations performed based upon known β-amyliod levels (Scheuner et al., *Nature Med. 2*: 864-870 (1996)) and a medium-range plasma level of bispecific antibody expected in a treated animal indicated 99.9% of the bispecific antibodies that enter the brain will have unoccupied anti-~~Aβ~~ β-amyloid combining sites.

Another way of delivering antibodies to the brain is via direct infusion of anti-β-amyloid antibodies into the brain of an animal. This technique gives these antibodies immediate access to β-amyloid in the brain without having to cross the blood-brain barrier. Direct infusion can be accomplished via direct parenchymal or intracerebroventricular infusion (Knopf et al., *J Immunol. 161*: 692-701 (1998)). Briefly, the animal is anesthetized and placed in a stereotaxic frame. A midsaggital incision is made on the scalp to expose the skull and the underlying fascia is scraped away. A hole is drilled to accept a sterilized length of stainless steel hypodermic tubing, which is

stereotaxically advanced so that its tip is appropriately located in the brain. A guide cannula is then attached to the skull and sealed. The cannula remains in place for multiple infusions of antibody into the brain. A bolus of a sterile 50 mg/ml solution of a monoclonal anti-β-amyloid can be infused over a 2-8 minute period into an immobilized animal via an injection cannula.

Delivery of catalytic antibodies into the brain of an animal via one of the above described methods, can also be used to disaggregate amyloid plaques present in the brain. The advantage of delivering an β-amyloid-specific catalytic antibody into the brain is two-fold. The β-amyloid peptide is permanently destroyed by such antibodies and, since catalysis is continuous, each antibody inactivates many target β-amyloid molecules in the brain. Thus much less vectorized bispecific antibody has to be delivered into the central nervous system to achieve the desired depletion of β-amyloid.

The amount of antibody to be administered or delivered to the animal should be sufficient to cause a significant reduction in β-amyloid levels in the brain of the animal. The appropriate amount will depend upon various parameters (e.g. the particular antibody used, the size and metabolism of the animal, and the levels of endogenous β-amyloid) and ~~should be~~ is to be determined on a case by case basis. Such determination is within the means of one of average skill in the art through ~~the course of~~ no more than routine experimentation.

It is expected that additional benefits with respect to lowering brain β-amyloid levels and preventing or disaggregating amyloid plaques can be achieved through utilizing a combination of one or more of the above described approaches.

Exemplification

SECTION

~~PART~~ 1: RETENTION OF β-AMYLOID IN THE CIRCULATION

Synthesis of β-Amyloid Peptide Antigens

The amino acid sequence of the 43 residue β-amyloid peptide (Aβ) is listed in Figure 1. To determine which sites on this Aβ peptide were best suited for antibody-mediated therapy, three key regions (amino-terminal, central and carboxy-terminal) of the Aβ 43-mer were chosen to generate epitope-specific vaccines. These shortened peptides served as antigenic epitopes to induce a highly specific antibody response.

Monoclonal antibodies to the amino-terminal region of Aβ have been shown in the past to have the ability to solubilize Aβ aggregates (Solomon et al., *Proc. Natl. Acad. Sci. USA* 94(8): 4109 (1997))(Solomon et al., *Proc. Natl. Acad. Sci. USA* 94(8): 4109 (1997); Solomon et al., *Proc. Natl. Acad. Sci. USA* 93(1): 452 (1996)). for the present experiments, A peptide consisting of the amino-terminal region of Aβ was similarly designed for the present experiments (shown in Fig. 2 and listed in SEQ ID NO: 2) and used to elicit amino-terminal specific antibodies that bind Aβ. A Cys residue was added to the C-terminus of the Aβ sequence to provide a suitable linkage group for coupling this peptide to an antigenic carrier protein such as maleimide-activated Keyhole Limpet Hemocyanin (KLH).

A peptide encompassing the central region of Aβ was also synthesized (shown in Fig~~ure~~ 3 and listed in SEQ ID NO: 3). A Cys residue was placed at the N-terminus of the Aβ sequence to provide a sulfhydryl linkage group for coupling the peptide to antigenic maleimide-activated carrier proteins such as KLH.

To produce an antigen for eliciting an immune response directed against the carboxy-terminus of Aβ (Suzuki et al., *Science* 264:1336(1994)), a decapeptide encompassing the N-terminal region of Aβ, with an additional Cys residue at the N-terminus, was synthesized (Shown in Fig. 4, and listed in SEQ ID

NO: 4). The Cys substitution was designed to provide a sulfhydryl linkage group for coupling the peptide to antigenic a ~~maleimide-activated~~ carrier proteins such as KLH.

Coupling the peptides to an antigenic carrier protein

The different Cys containing Aβ peptides were individually thioether-linked to maleimide-activated KLH. A multivalent Aβ vaccine was also produced by simultaneously linking all three of these peptides to maleimide-activated KLH. In addition the full-length Aβ 43-mer was linked to KLH using glutaraldehyde.

Antibodies Elicited with the β-Amyloid Vaccines

Normal BALB/c mice were immunized by standard procedures with the KLH-linked Aβ vaccines described above. The mice were either bled or sacrificed for removal of the spleen for hybridoma production. Sera and monoclonal antibodies obtained were characterized for binding to Aβ.

Table 1 shows the results from an ELISA run with 1/100 diluted serum from two non-immunized control mice versus 1/100 and 1/1000 diluted serum from a mouse that was immunized with a central region Aβ peptide-KLH vaccine. The free Aβ peptide was adsorbed directly onto the microtitre plate to avoid detection of anti-KLH ~~antibodies in~~ the serum. Monoclonal antibodies raised have also been ~~against this central region Aβ peptide~~ ~~[illegible] have also been~~ ~~successfully identified~~ using this ELISA assay. and produced 2D6 by hybridoma fusion were identified

okay

Table 1 ELISA for Binding to the Central Region Aβ Peptide

Addition		Antibody Bound (O.D. 450nm)
Control Serum A	1/100	0.666
Control Serum B	1/100	0.527
Mouse 1 antiserum	1/100	3.465

Mouse 1 antiserum 1/1000 2.764

A binding assay was performed to determine whether the monoclonal anti-$A\beta$ antibodies identified ~~by the above assays~~ also bound to the full length $A\beta$ peptides. ^{125}I-$A\beta_{1-43}$ probe was incubated with hybridoma secretions from the indicated clones. A standard polyethylene glycol separation method was used to detect ^{125}I-$A\beta_{1-43}$ bound antibody (Table 2). Results presented in Table 2 indicate that the antibodies generated to the peptide fragments also bound full length $A\beta_{1-43}$.

Table 2 ^{125}I-$A\beta_{1-43}$ Binding Assay

Addition		^{125}I-$A\beta_{1-43}$ Bound (cpm)
Control Hy		3,171
Control Hy		2,903
6E2		15,938
6E2	1/10	9,379
3B1		12,078
3B1	1/10	3,353
8E3		10,789
8E3	1/10	3,249

This raises the concern that ~~This reported result~~ ~~suggests that~~ the ~~albumin~~ bound albumin will interfere w/ anti-body binding.

It was recently reported that when ^{125}I-$A\beta_{1-40}$ is added to human plasma, ~89% binds to albumin (Biere et al., *Journal of Biological Chemistry* 271(51):32916 (1996)). Binding assays were performed in the presence and absence of serum albumin, to determine whether albumin binding ~~would~~ interferes with antibody

binding to Aβ. The ability of purified 5A11 monoclonal anti-Aβ antibody to bind ^{125}I-$A\beta_{1-40}$ was unaffected by the presence of human serum albumin (HSA) at 60 mg/ml, even though this was a 500-fold molar excess over the antibody concentration (Table 3). These results indicate that the ability of antibodies to bind to and sequester Aβ in the blood will not be attenuated by the presence of other binding proteins.

Table 3. ^{125}I-$A\beta_{1-40}$ Binding to Antibody in the Presence of Human Serum Albumin*

Addition	^{125}I-$A\beta_{1-40}$ Bound (cpm)	Specifically Bound (% of total added)
Control	8,560	-
+ 5A11 anti-Aβ	64,589	79
Control + HSA*	3,102	-
+ 5A11 anti-Aβ + HSA*	55,304	75

*HSA at 60 mg/ml (~1 mM); anti-Aβ 5A11 at $2x10^{-6}$ M; Added ~70,000 cpm of ^{125}I-$A\beta_{1-40}$

analog at an amide linkage)

discussed further in section II

Monoclonal Antibody Production

had a

This peptide also possessed a analog

A mouse was immunized with a KLH conjugate of the central region phenylalanine statine transition state ~~mimic~~ of the central region $A\beta_{10-25}$ peptide. A hybridoma fusion was performed and the resulting monoclonal antibodies analyzed to characterize the specificity of the immune response to the vaccine. Hybridoma supernatants produced in the fusion were screened using ELISA to assess their binding to the $A\beta_{1-43}$ peptide.

The monoclonal antibodies produced were determined to bind to the $A\beta_{1-43}$ peptide adsorbed directly onto an ELISA plate. Strong color reactions were obtained in this ELISA using only 10 μl of hybridoma supernatant while the addition of media alone produced low background color. These results indicate that the

antibodies not only bound to the small peptide immunogen but they were also reactive with the full-length $A\beta_{1-43}$. Importantly, antibodies bound to the carrier-free $A\beta$ peptide adsorbed directly onto microtitre plates, showing their specificity for the peptide rather than the immunogenic carrier. The high affinity 5A11 monoclonal antibody (Table 3) was obtained from this hybridoma fusion. **[VR: THIS IS MISLEADING. PLEASE RECTIFY THIS STATEMENT WITH THE FACT THAT ANTIBODY 5A11 WAS OBTAINED FROM IMMUNIZATION WITH A TRANSITION STATE MIMIC PEPTIDE ANTIGEN.]**

A second mouse was immunized with a KLH conjugate of the $A\beta_{35-43}$ analog encompassing the C-terminal region of $A\beta$. Serum from the mouse was screened for reaction with $A\beta_{1-43}$ adsorbed directly onto the ELISA wells. The assay results are presented in Table 4. The spleen of this mouse was then used for a hybridoma fusion to further characterize the specificity of its immune response. Importantly, none of the mice immunized with $A\beta$ vaccines or the anti-$A\beta$ ascites-producing mice displayed ill effects even though some of those induced antibodies cross-react with mouse $A\beta$ and mouse amyloid precursor protein.

Table 4 ELISA for Binding of Antiserum Directed to the Carboxy-terminal $A\beta$ Peptide

	Antibody Bound (O.D. 450nm)
Addition	**Native $A\beta_{1-43}$**
Control Serum	0.484
Mouse Antiserum	1.765

Monoclonal antibodies from hybridoma clones generated above were screened for binding to the small carboxy-terminal peptide $A\beta_{35-43}$ and the full-length $A\beta_{1-43}$. Results are presented in Figure 5. The monoclonal antibodies bound to the carboxy-

terminal locus on each of these carrier-free Aβ peptides adsorbed directly to the microtitre plate, confirming their specificity for the peptide rather than the immunogenic carrier. The clones were also tested with Aβ_{1-40} to identify antibodies which do not react with this shortened, 40 amino acid residue version of Aβ and thus will specifically bind to the carboxy-terminus of Aβ_{1-43} (Fig.5). Used therapeutically, this vaccine should elicit antibodies which will preferentially bind the less abundant but more noxious Aβ_{1-43} species in the blood, as opposed to the smaller and less detrimental Aβ_{1-40}.

<u>Antibodies Affect the Distribution of ^{125}I-Aβ in Normal Mice</u>

Anti-Aβ antibodies in the circulation cannot cross the blood-brain barrier to a significant extent and therefore should act as a sink that prevents ^{125}I-Aβ_{1-40} from reaching the brain. This retention effect was demonstrated by measuring the blood levels in mice 4 h after injecting them with equal amounts of ^{125}I-Aβ_{1-40} either alone or along with our 5A11 anti-Aβ monoclonal antibody (Table 5). The passage of ^{125}I-Aβ_{1-40} out of the peripheral circulation was greatly curtailed in animals which concomitantly received the specific anti-Aβ antibody. That finding extends the *in vitro* results with the 5A11 antibody (Table 3) by demonstrating that it can also effectively bind Aβ in an experimental animal. The observation ~~fact~~ that animals treat~~ment~~ed with this antibody led to the ~~retained~~ 10-times more ^{125}I-Aβ_{1-40} in the circulation indicates that the equilibrium distribution of Aβ in the body can be dramatically altered by ~~125~~ selective sequestration in the blood.

Table 5 Anti-Aβ Antibody Impedes the Passage of ^{125}I-Aβ_{1-40} Out of the Circulation

^{125}I-Aβ_{1-40} in Blood

Mouse Injected With	**(cpm/gm)**
^{125}I-$A\beta_{1-40}$ alone	27,300
^{125}I-$A\beta_{1-40}$ + 5A11 anti-$A\beta$	278,900

Materials and Methods

Peptide synthesis. The 40mer $A\beta_{1-40}$, the 43mer $A\beta_{1-43}$, and the three small $A\beta$ peptides $A\beta_{1-16}$, $A\beta_{10-25}$, and $A\beta_{35-43}$, were synthesized by standard automated Fmoc chemistry. Newly synthesized peptides were purified by HPLC and their composition was verified by mass spectral and amino acid analysis. The $A\beta$ 43mer was obtained from a commercial source (Bachem, Torrance, CA).

Conjugation of β-amyloid peptides to immunogenic carriers. The small $A\beta$ peptides were linked to the KLH carrier protein in order to render them antigenic. A Cys residue was strategically placed at the N- or C-terminal end of these $A\beta$ peptides to provide a suitable linkage group for coupling them via a thioether bond to maleimide activated carrier proteins. This linkage is stable and attaches the peptide in a defined orientation. Addition of ~20 peptides/KLH is typically obtained by this conjugation method. The longer, full length $A\beta$ peptides were linked to carrier proteins using a glutaraldehyde coupling procedure. ~~The outlined methods are an effective and expedient way of producing experimental vaccines for use in animals.~~

Immunization of Mice. Normal BALB/c mice were immunized by standard procedures with the KLH-linked $A\beta$ vaccines described above. Briefly, mice were injected i.p. with antigen emulsified in complete Freunds adjuvant, followed by a second course in incomplete Freunds adjuvant. The mice were i.v. boosted with antigen in PBS three days prior to bleeding them or removing the spleen for hybridoma fusions to produce monoclonal antibodies.

None of the mice immunized with Aβ vaccines or the anti-Aβ ascites-producing mice displayed ill effects even though some of th~~ese~~e ~~induced~~ antibodies cross-reacted with mouse Aβ and mouse amyloid precursor protein.

ELISA. The presence of bound anti-peptide antibodies was revealed by using a peroxidase-labeled anti-mouse IgG probe followed by the chromogenic substrate (Engvall et al., W Immunochemistry 8: 871-875 (1971).

Binding Assay. Both $A\beta_{1-43}$ and $A\beta_{1-40}$ were radiolabeled with ^{125}I. The iodinated peptide was separated from unlabeled material by HPLC to give essentially quantitative specific activity (~2000 Ci/mmol) (Maggio et al., *Proc. Natl. Acad. Sci.* 89:5462 (1992)). ^{125}I-$A\beta_{1-43}$ probe was incubated for 1h at 23°C with Hy media taken from hybridoma clones producing monoclonal anti-Aβ antibodies. A standard polyethylene glycol separation method was used to detect the amount of ^{125}I-$A\beta_{1-43}$ bound to antibody.

Section ~~PART~~ II: ELICITING MONOCLONAL ANTIBODIES WITH TRANSITION STATE ANTIGENS

Transition state peptide antigens

Different types of transition state peptide antigens were synthesized to use in the generation of antibodies which preferentially recognize (hydrolysis) transition states of Aβ at a predetermined amide linkage position.

A series of statine (Sta) transition state analogs encompassing the carboxy-terminal region of Aβ (Cys-Met-Val-Gly-Gly-Val/Sta-Val/Sta-Ile/Sta-Ala-Thr) were synthesized. Replacement of the proposed scissile peptide linkage between Val_{39} and Val_{40} ~~(4)~~, Val_{40} and Ile_{41} ~~(5)~~ and Ile_{41} and Ala_{42}, ~~(6)~~ with a "statyl" moiety ($-CHOH-CH_2-CO-NH-$) was designed to elicit

catalytic antibodies that hydrolytically cleave Aβ at one of these sites (Figure 6). A Cys residue was placed at the N-terminal position of these peptides to provide a suitable linkage group for coupling to a maleimide-activated carrier protein.

A series of phenylalanine statine (PhSta) transition state analogs encompassing the central region of Aβ (Cys-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe/PhSta-Phe/PhSta-Ala-Glu-Asp-Val-Gly-amide) was synthesized in this laboratory (Fig. 7).

Replacement of the proposed scissile peptide linkage between Phe_{19} and Phe_{20}, and between Phe_{20} and Ala_{21} with a statyl moiety (-CHOH-CH_2-CO-NH-) was designed to elicit catalytic antibodies that hydrolytically cleave Aβ at these sites (Figure 7). A Cys residue was placed at the C-terminus of these peptides to provide a sulfhydryl linkage group for coupling the peptides to antigenic, maleimide-activated carrier proteins ~~such as KLH~~.

A structural comparison (Fig. 8) was made between the native Aβ peptide and the transition state phenylalanine statine Aβ peptide using a graphics workstation. An energy minimization algorithm (2000 iterations) was applied to arrange each peptide in its most favorable conformation.

The peptide link -CO-NH- between Phe_{19} and Phe_{20} was replaced with an elongated "statyl" moiety -CHOH-CH_2-CO-NH- and an energy minimization was applied. This orientation shows the difference between the planar peptide link -CO-NH- of natural Aβ (left) versus the extended, tetrahedral "statyl" moiety -CHOH-CH_2-CO-NH- in the transition state peptide (right).

An antibody combining site complementary to a tetrahedral statine transition state analog will force the planar peptide bond of the Aβ substrate into a transition state-like conformation. Such distortion should catalyze the cleavage of Aβ at that locus in the peptide sequence.

A reduced peptide bond linkage can be easily placed at almost any site in the Aβ molecule to produce a reduced peptide bond transition state analog. This analog can also be used to

The possibility of using a reduced peptide bond linkage to mimic the ~~hydrolysis~~ transition state of hydrolysis of ~~the~~ an amide ~~peptide bond~~ linkage was also explored

elicit catalytic antibodies that will hydrolytically cleave Aβ at the chosen site. The ~~first~~ reduced peptide bond transition state Aβ analog made was the (Gln-Lys-Leu-Val-Phe-CH_2-NH_2^+-Phe-Ala-Glu-Asp-Val-Gly-Cys-amide) central region peptide; [calculated 1,342 (M+1); observed 1,344].

A structural comparison (Fig. 9) was made between the native Aβ peptide and the reduced peptide bond transition state Aβ analog using a graphics workstation. An energy minimization algorithm (2000 iterations) was applied to arrange each peptide in its most favorable conformation. The peptide link -CO-NH- between Phe_{19} and Phe_{20} was replaced with a reduced peptide bond -CH_2-NH_2^+- and an energy minimization was applied. The orientation shown indicates the difference between the planar peptide link -CO-NH- of natural Aβ (left) versus the corresponding tetrahedral moiety -CH_2-NH_2^+- in the reduced peptide bond transition state analog (right).

A phosphonamidate transition state analog of the carboxy-terminal region of Aβ has also been synthesized (Fig. 10). Replacement of the proposed scissile peptide linkage between Gly_{38} and Val_{39} with a phosphonamidate moiety (-PO_2^--NH-) was designed to elicit catalytic antibodies that will hydrolytically cleave Aβ at this site. The N-acetyl-Cys residue was placed at the position of Leu_{34} to provide a suitable linkage group for coupling this peptide to an antigenic carrier protein. The structures in Fig. 11 represent the putative transition state for peptide hydrolysis by zinc peptidases, ~~and~~ versus structure of the phosphonate and phosphonamidate mimics. Similar tetrahedral transition state intermediates are known to be formed by reaction with each of the four classes of proteolytic enzymes, the serine-, cysteine-, aspartic- and metallo-peptidases.

The synthesis of phosphonate Aβ transition state analog peptide (eg. N-acetyl-Cys-Met-Val-Gly-Gly-PO_2^--O-Val-Val-Ile-Ala-amide) will follow a similar scheme and will use some of the same intermediates described for the phosphonamidate transition state

analog.

A structural comparison was made between the native Aβ peptide and the transition state phosphonamidate Aβ peptide (Fig. 12) using a graphics workstation. The peptide link -CO-NH- between Gly_{38} and Val_{39} was replaced with a phosphonamidate bond -PO_2^--NH- and an energy minimization was applied. The orientation shown in Fig. 12 illustrates the difference between the planar peptide link -CO-NH- of native Aβ (left) versus the corresponding tetrahedral phosphonamidate bond -PO_2^--NH- in the transition state peptide (right).

An antibody combining site complementary to the tetrahedral transition state analog on the right of Fig. 12, will force the normally planar bond of the Aβ substrate peptide on the left into a transition state-like conformation. Such bond distortion was expected to catalyze the hydrolytic cleavage of the Aβ peptide at the Gly_{38}-Val_{39} linkage.

Immunization with transition state peptide antigens

Peptide antigens were coupled to the immunogenic carrier KLH prior to immunization of mice.

Standard protocols were used to immunize the Tg2576 transgenic mice and BALB/c mice with the KLH-linked Aβ peptides described in the preceding sections. Briefly this procedure used i.p. injection of the different antigens emulsified in complete Freunds adjuvant, followed by a second course in incomplete Freunds adjuvant. Three days prior to hybridoma fusion, the BALB/c mice were boosted i.v. with antigen in PBS.

A hybridoma fusion was performed using the spleen of a mouse immunized with the phenylalanine statine transition state Aβ-KLH antigen (Fig. 7) and also the statine (Sta) transition state analogs encompassing the carboxy-terminal region of Aβ (Fig. 6) (Cys-Met-Val-Gly-Gly-Val/Sta-Val/Sta-Ile/Sta-Ala-Thr) **[SH:THESE WERE NOT THE ONLY TRANSITION STATE ANTIGENS USED TO GENERATE HYBRIDOMAS/MONOCLONAL ANTIBODIES. VR IS MAKING A TABLE CATALOGING**

THE DIFFERENT TS ANALOGS, SITES MODIFIED AND MABS GENERATED]

Demonstration of Aβ binding by generated antibodies

It was very important to demonstrate that the anti-Aβ and anti-transition state Aβ monoclonal antibodies bound to the natural $A\beta_{1\text{-}43}$ peptide which they were designed to sequester or cleave. To do this, $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}43}$ were radiolabeled with ^{125}I and the iodinated peptides were ~~then~~ separated from unlabeled material by HPLC. Probe was incubated with either purified anti-Aβ antibodies or media taken from hybridoma clones producing anti-Aβ antibodies. The amount of $^{125}I\text{-}A\beta_{1\text{-}43}$ bound to antibody was determined using a polyethylene glycol separation method. Results of the experiment are presented in Table 6.

The data in Table 6 demonstrate the ability of the purified 5A11 monoclonal anti-Aβ antibody to bind a high percent of $^{125}I\text{-}A\beta_{1\text{-}40}$. This binding assay was used to screen clones and purified antibodies (Table 6) for their ability to bind Aβ (~~below~~). Similar procedures can also serve as the basis for a competitive displacement assay to measure the relative binding strength of different unlabeled Aβ peptides (Note: With very efficient catalytic antibodies this binding assay may have to be performed on ice to ensure that no cleavage of Aβ occurs during the 1h incubation time.) The assay ~~will~~ allows the rapidly identified ~~ication of~~ clones ~~which~~ produce high affinity anti-Aβ antibodies.

Table 6 **$^{125}I\text{-}A\beta_{1\text{-}40}$ Binding to a Purified Monoclonal Anti-Aβ Antibody ***

Addition	$^{125}I\text{-}A\beta_{1\text{-}40}$ Bound (cpm)	Specifically Bound (% of total added)
Control	8,560	-
+ 5A11 anti-Aβ	64,589	79

* anti-Aβ 5A11 at $2x10^{-6}$ M; Added ~70,000 cpm of ^{125}I-Aβ_{1-40}

Monoclonal antibodies from ~~some of~~ hybridomas ~~supernatants~~ obtained using the phenylalanine statine transition state Aβ-KLH antigen were screened ~~using~~ by ELISA to assess their binding to both the normal Aβ_{1-43} peptide and to the phenylalanine statine transition state Aβ peptide. Two major patterns were found (Fig. 13).

One group of antibodies (the left portion of Fig. 13) bound to the immunizing transition state peptide and cross-reacted strongly with the native Aβ_{1-43} peptide (~~when~~ each was adsorbed directly onto the ELISA plate). ~~A~~ The second group (the right portion) showed a high binding preference for the phenylalanine statine transition state Aβ peptide and reacted minimally with native Aβ_{1-43}.

Strong color reactions were obtained in this ELISA using only 10 μl of hybridoma supernatant while Hy media alone or PBS gave a low background (Fig. 13). These results demonstrate that the comparative ELISA screen, although only a semi-quantitative measure of binding, provides a means for ~~choosing~~ identifying monoclonal antibodies that are highly selective for, and most reactive with, the transition state. Importantly, experiment was performed with ~~the antibodies bound to the~~ carrier-free Aβ peptides adsorbed directly onto microtitre plates, indicating antibody ~~showing their~~ specificity for ~~the~~ peptide, rather than the carrier

These findings indicate that several of the generated anti-Aβ transition state antibodies were unique. They bound to both the phenylalanine statine- and normal-Aβ peptides. Their selective recognition of the transition state and weaker cross-reaction with native Aβ_{1-43} however, indicates that this binding interaction is very different from that shown by conventional anti-native Aβ antibodies. It further indicates that these new antibodies may be able to force the native Aβ peptide into a conformation resembling the transition state for hydrolytic cleavage. Importantly, some of the antibodies which showed only

minimal binding to $A\beta_{1-43}$ in this ELISA, did display cross-reactivity with the natural peptide using a highly sensitive ^{125}I-$A\beta_{1-43}$ binding assay (Table 6).

ELISAs were also performed to investigate the binding of anti-statine analog antibodies to both the normal $A\beta_{1-43}$ peptide and to the statine transition state $A\beta$ peptide (Fig. 14). The antibodies bound to the C-terminal locus on these carrier-free $A\beta$ peptides (adsorbed directly to the microtitre plate), confirming their anti-peptide specificity. Most of the antibodies preferentially recognized the statine $A\beta$ transition state but cross-reacted with native $A\beta_{1-43}$. This indicates that these new antibodies are able to force the native $A\beta$ peptide into a conformation resembling the transition state for hydrolytic cleavage of its C-terminal amino acids. Such cleavage is predicted to convert $A\beta_{1-43}$ into potentially less harmful shorter peptides, like $A\beta_{1-40}$ or $A\beta_{1-39}$.

Clone 11E9 had the strongest preference for the statine analog and may be the most likely to have catalytic activity (Fig. 14). Several clones displayed no difference in their reactivity with the native versus statine transition state $A\beta$ peptide. The clones were also tested with $A\beta_{1-40}$ to identify antibodies which do not react with this shortened, 40 amino acid version of $A\beta$ (Fig. 14). Used therapeutically, such antibodies should preferentially bind/cleave the less abundant but more noxious $A\beta_{1-43}$ species in the blood, as opposed to the smaller and less detrimental $A\beta_{1-40}$.

Solid phase and TLC $A\beta$ proteolytic assays

A solid phase ^{125}I-labeled $A\beta$ assay was developed to screen anti-transition state antibody hybridoma supernatants for specific proteolytic activity. The peptide Cys-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Tyr-amide (SEQ ID NO: 5) which encompasses amino acids 14-25 of $A\beta$ was radiolabeled and coupled

to a thiol-reactive, iodoacetyl-Sepharose gel to form an irreversible linkage. The product was contacted with anti-transition state antibody and assayed for the progressive release of soluble ^{125}I-peptide from the solid phase matrix. Release of radioactivity from the ^{125}I-Aβ-Sepharose was used to identify catalytic activity (Fig. 15). The assay was verified by the ability of several different proteases to rapidly hydrolyze this Sepharose-linked Aβ substrate. The peptide was readily accessible to proteolytic cleavage as revealed by a release of soluble ^{125}I-peptide that increased with incubation time.

The results presented in Figure 15 indicate that the antibody-containing media of several clones released ^{125}I-peptide at a greater rate than other clones from this fusion or the PBS and Hy medium controls. Large amounts of these antibodies can be obtained, purified and tested at higher concentrations to achieve much faster rates of cleavage and to verify that the antibodies are acting in a catalytic mode using conventional enzyme kinetics. By changing the composition of the ^{125}I-peptide this same strategy can be used to assay antibodies reactive with different regions of Aβ.

A thin layer chromatography-based autoradiography assay was devised to obtain more definitive evidence for antibody-mediated cleavage of Aβ. Selected anti-phenylalanine statine Aβ transition state clones were expanded and ascites production induced. The different monoclonal antibodies were isolated using protein A-Sepharose. Two ^{125}I-labeled peptides, ~~^{125}I~~ Aβ_{1-40} and a 17-mer, encompassing amino acids 9-25 of Aβ, were used to test for peptide cleavage. The antibodies were added to the ^{125}I-peptides, allowed to incubate and the reaction mix spotted onto polyamide thin layer sheets which were then developed in different solvents. The migration of ^{125}I-products was followed by exposing the sheet using a quantitative phosphoimager system. Quantitation of the different ~~sized~~ labeled peptide fragments produced indicated that addition of the antibodies to the Aβ

peptides lead to significant break down of the Aβ peptides compared to the untreated peptides (PBS).

Disaggregation of β-amyloid by monoclonal antibodies has been shown previously to

The self-aggregation of synthetic Aβ peptides ~~leads~~ to microscopic structures resembling amyloid plaques in the brain (Solomon et al., *Proc. Natl. Acad. Sci. USA* 94: 4109-12 (1997); Solomon et al., *Proc. Natl. Acad. Sci. USA* 93: 452-5 (1996)) which exhibit the same bright green fluorescence upon exposure to thioflavin T. These aggregates are very stable and usually require harsh detergents or strong acids to dissolve. However, it has been demonstrated that the binding of certain anti-Aβ monoclonal antibodies can effectively inhibit the initial aggregation of this peptide and also disaggregate preformed Aβ complexes (Solomon et al., *Proc. Natl. Acad. Sci. USA* 94: 4109-12 (1997); Solomon et al., *Proc. Natl. Acad. Sci. USA* 93: 452-5 (1996)).

A radioactive assay was used to quickly screen the different monoclonal antibodies ~~produced~~ produced by the present experiments for an ability to dissolve preformed Aβ aggregates, made with ^{125}I-labeled and unlabeled soluble Aβ peptide. An aliquot of the labeled aggregate was incubated with either PBS, the 5A11 anti-Aβ antibody, or an equal amount of an irrelevant mouse antibody (7D3, anti-human transferrin receptor), and the level of released radioactivity was subsequent measured (Table 7). ~~The fact that~~ the Aβ-specific 5A11 antibody solubilized 80% of the Aβ aggregates while an equal amount of the control antibody had only a minor effect, suggests that the equilibrium was displaced by antibody-mediated binding of soluble Aβ.

Table 7 Solubilization of ^{125}I-Aβ_{1-40} Aggregate by Monoclonal Anti-Aβ Antibody

Addition	^{125}I-Aβ_{1-40} in Ppt. (cpm)	Amount Solubilized (% of PBS Control)

PBS control	3,420	-
+ 5A11 anti-Aβ	676	80
+ 7D3 anti-TfR	2,458	27

Production of Vectorized Anti-Aβ/Anti-Receptor Bispecific Antibodies

Anti-Aβ antibodies were linked to anti-transferrin receptor antibodies (anti-TfR) which served as vectors for delivery of the anti-Aβ antibodies into the brain. The 7D3 mouse monoclonal antibody was used as the anti-TfR part of the construct. 7D3 is specific for the human receptor and selectively immunostains cortical capillaries in normal human brain tissue (Recht et al., *J Neurosurg* 72: 941-945 (1990)). Antibody attachment to the receptor is not blocked by an excess of human transferrin. The epitope recognized by this antibody is therefore distant from the receptor-ligand binding site. Bispecific antibodies constructed with this 7D3 antibody and an anti-Aβ antibody are predicted to be useful for therapy in patients with Alzheimer's disease. ~~Stained with the 7D3 Anti-TfR (Recht et al., *J Neurosurg* 72:941-945 (1990)) and possibly for preclinical trials in primates.~~

For studies in the transgenic mouse model of Alzheimer's disease an anti-mouse transferrin receptor monoclonal antibody produced in the rat was obtained. This antibody also appears to recognize a transferrin receptor epitope which does not involve ligand binding. The antibody therefore has no effect on cell proliferation when ~~tested~~ using murine lines.

A series of functional assays were performed after completion of the synthesis, purification and size analysis of the anti-Aβ/anti-transferrin receptor bispecific antibodies. The vectorized bispecific antibody, composed of a rat monoclonal antibody directed against the mouse transferrin receptor plus the 5A11 mouse anti-Aβ monoclonal antibody, was tested for the ability to attach to transferrin receptor bearing human cells.

Both components of the bispecific antibody were detected on the cell membrane by cytofluorimetry (Fig. 16) when this duplex was reacted with transferrin receptor positive mouse cells and probed using either a rat IgG-specific or mouse IgG-specific fluorescent secondary antibody reagent.

The capacity of the hybrid reagent to bind ^{125}I-Aβ compared favorably with that of the parent anti-Aβ antibody (Table 8).

Table 8 **^{125}I-Aβ Binding to Bispecific Antibody**

Addition	^{125}I-Aβ_{1-40} Bound (cpm)
Control	4,199
+ anti-Aβ	23,301
+ anti-Aβ/anti-receptor	22,850

To ensure that both of these binding activities resided on the bispecific antibody, transferrin receptor positive cells were treated with the hybrid reagent, unbound material was washed away, and then the cells with bound antibody was exposed to ^{125}I-Aβ_{1-40}. After washing away unbound Aβ, the cell-bound radioactivity was compared to control cells which had been identically prepared except for omission of pretreatment with bispecific antibody. The results are presented in Table 9, and verify the dual specificity of this bispecific antibody by clearly showing that it can simultaneously attach to the cell membrane and bind ^{125}I-Aβ_{1-40}.

Table 9 Bispecific Antibody-Mediated Binding of ^{125}I-Aβ to Receptor-Positive Cells

Pretreatment of Cells	^{125}I-Aβ_{1-40} Bound (cpm)
None	2,367
+ anti-Aβ/anti-transferrin receptor	11,476

Transcytosis of bispecific antibody into the brain

A rat monoclonal anti-mouse transferrin receptor antibody was coupled to a mouse monoclonal antibody (obtained from American Type Culture Collection (ATCC TIB 219), also designated R17 217.1.3 (Cell. Immunol. 83: 14-25 (1984))) so that the entry of this new vectorized bispecific construct into brain could be monitored. The bispecific antibody was labeled with ^{125}I and injected i.v. into normal mice. After different lengths of time the mice were sacrificed and the amount of ^{125}I-bispecific antibody that crossed the blood-brain barrier and entered the brain was gauged by a mouse capillary depletion method (Friden et al., *J. Pharm. Exper. Ther.* 278:1491-1498 (1996); Triguero et al., *J. Neurochem.* 54: 1882-1888 (1990)).

The amount of vectorized bispecific antibody found in the brain parenchyma or brain capillary fractions was measured following differential density centrifugation of the brain homogenate. These values were plotted as a function of time after i.v. injection (Fig. 17). The time-dependent redistribution of radiolabeled bispecific antibody from the capillaries and into the parenchyma was consistent with its passage across the cerebral endothelial blood-brain barrier (Joachim et al., *Nature* 341: 6239:226-30 (1989)). Even greater accumulation in the parenchyma is expected to occur if the antibodies attach to Aβ in the cerebral plaques of plaque-bearing mice.

Monitoring the brain distribution of bispecific antibody in live mice

The ability to follow the entry and accumulation of vectorized bispecific antibodies in the brain of live mice would greatly assist in the development of the intracerebral treatment of plaque-bearing mice. Such a development would enable time-course studies and would greatly reduce problems with inter-mouse variability. Preliminary studies with ^{125}I-labeled bispecific antibodies were performed to determine if immunoscintigraphy was feasible in this system. As a first step, either the radiolabeled vectorized bispecific antibody (^{125}I-R17/5A11) or a non-vectorized control bispecific antibody were administered i.v. to separate mice. Sequential brain images were accumulated at 1, 6, 24 and 48 hours following i.v. administration of the ^{125}I-labeled bispecific antibody probes. Although this technique suffered from a difficulty in determining how much of the signal was due to the levels of blood-borne radioactivity circulating through the brain, significant distinctions were noted in the brain of mice treated with the mouse transferrin receptor reactive bispecific antibody versus those receiving the control bispecific antibody. When the vectorized agent was used, brain levels increased between 1 and 6 hrs and then declined to a much lower level at 24 and 48 hrs. Mice treated with the control displayed no increase between 1 and 6 hrs. The reason for decreased brain levels at 24 hrs and beyond is not known but might be due to dehalogenation of the bispecific antibody probes so that free ^{125}I is released ~~which exits the brain~~. Alternative methods utilizing radioactive labels such as ^{111}In (Sheldon et al., *Nucl. Med. Biol.* 18:519-526 (1991)) or ^{99m}Tc (Texic et al., *Nucl. Med. Biol.* 22:451-457 (1995)) attached to the vectorized bispecific antibody can be utilized in future experiments if the use of iodine presents a technical problem. This imaging technology will be useful for determining if smaller vectorized bispecific antibodies (eg. $F(ab')_2$) with different physical properties and an altered

biodistribution will penetrate into the brain more effectively.

F(ab')$_2$ heterodimers for vector-mediated transport into the brain

The introduction of whole antibodies into the brain might be detrimental if they were to fix complement and promote complement-mediated lysis of neuronal cells. The development of smaller vectorized F(ab')$_2$ bispecific reagents is expected to avoid this problem. It has been shown that aggregated Aβ itself can fix complement in the absence of any antibody and that the resulting inflammation may contribute to the pathology of Alzheimer's disease. The possibility of intracerebral antibody having a similar effect would be greatly reduced by eliminating the Fc region of the antibody. Moreover, since coupling of Fab' halves uses the intrinsic hinge region cysteines, no extraneous substituent linkage groups need ~~to~~ be added.

Faster or more efficient entry into the brain represents another potential advantage that smaller F(ab')$_2$ or Fv$_2$ reagents provide for intracerebral delivery. Such modified bispecific agents can be prepared and compared to ~~with~~ full-sized hybrid antibodies for the~~se~~ relative effectiveness in reaching the brain, crossing the blood-brain barrier, and affecting Aβ plaque development, by the methods described herein. It is important to note however that only minor differences were found when the capacity of differently-sized anti-transferrin receptor bispecific reagents for delivering toxins into cells by receptor-mediated endocytosis was compared (Raso et al., *J. Biol. Chem.* 272: 27623-27628 (1997)). This observation might indicate that little variation will be seen for transcytosis across the brain capillary endothelial cells which form the blood-brain barrier. At the very least however one would expect the two types of vectorized molecules to have different biodistribution and plasma half-life characteristics (Spiegelberg et al., *J. Exp. Med.* 121: 323 (1965)).

Materials and Methods

Antigen synthesis. The statine and phenylalanine statine transition state peptides were synthesized using automated Fmoc chemistry. Fmoc-statine (Sta), [N-Fmoc-(3S,4S)-4-amino-3-hydroxy-6-methyl heptanoic acid] and Fmoc-"phenylalanine statine" (PhSta), [N-Fmoc-(3S,4S)-4-amino-3-hydroxy-5-phenylpentanoic acid] were purchased commercially. Each peptide was tested for purity by HPLC and its composition was verified by mass spectral and amino acid analysis. The design strategy and methods for synthesizing phosphonamidate- and phosphonate-based transition state peptides are straightforward (Bartlett et al., *Am. Chem. Society* 22:4618-4624 (1983); Bartlett et al., *Biochemistry* 26:8553-8561 (1987)). The N-terminal portion of the peptide (N-acetyl-Cys-Met-Val-Gly) was made using standard automated Fmoc chemistry. After cleavage from the resin the N-acetyl tetrapeptide was treated with pyridine disulfide to protect its sulfhydryl group. An acid chloride of Cbz-glycine phosphonate monomethyl ester (Bartlett et al., *Am. Chem. Society* 22:4618-4624 (1983); Bartlett et al., *Biochemistry* 26:8553-8561 (1987)) was coupled with Val-Val-Ile-Ala-amide which was synthesized by automated Fmoc chemistry. The last amino acid of Aβ, Thr, was omitted due to potential problems with its unprotected hydroxyl group. The product, Cbz-Gly-PO_2^--NH-Val-Val-Ile-Ala-amide has a phosphonamidate (methyl ester) bond between the Gly and Val residues. Next, the Cbz blocking group was removed using hydrogen so that the protected N-acetyl-Cys-Met-Val-Gly peptide could be added to the amino terminal end of this transition state peptide by HBTU-activated peptide linkage. Treatment with mercaptoethanol and rabbit liver esterase was used to deblock the peptide. Each key component in the synthetic scheme was tested for purity by HPLC and its composition was verified by mass spectral and amino acid analysis.

¶ A reduced peptide bond linkage was placed at the indicated sites in the Aβ molecule. Automated Fmoc chemistry was used to begin synthesis of the peptide. A

pre-synthesized Fmoc amino aldehyde was then added manually and after the imide was reduced, automated synthesis was resumed (Meyer et al., *J. Med. Chem.* 38:3462-3468 (1995)).

<u>Coupling of antigen to carrier</u>. The native and transition state Aβ peptides were coupled to maleimide-activated KLH by standard procedures (Partis et al., J. Pro. Chem 2: 263-277 (1983), in order to elicit an immune response. A Cys residue was strategically placed at the N- or C-terminal end of the peptides to provide a suitable linkage group for coupling them via a thioether bond to maleimide activated carrier proteins. This stable linkage attaches the peptide in a defined orientation. Addition of ~20 peptides/KLH has been obtained based upon the transition state amino acid content as determined by amino acid analysis of the hydrolyzed conjugates (Tsao et al., *Anal. Biochem. 197*: 137-142 (1991)).

<u>Immunization of mice</u>. Standard protocols were used to immunize the Tg2576 transgenic mice and BALB/c mice with the KLH-linked Aβ peptides described in the preceding sections. Briefly this procedure used i.p. injection of the different antigens emulsified in complete Freunds adjuvant, followed by a second course in incomplete Freunds adjuvant. Three days prior to the hybridoma fusion, the BALB/c mice were boosted i.v. with antigen in PBS.

Aβ antigens will be emulsified in complete Freunds adjuvant and injected i.p. into BALB/c mice. After ~1 month animals were given a boost i.p. using the antigen emulsified with incomplete adjuvant. Serum from these animals was analyzed for anti-peptide antibodies by ELISA. BALB/c mice showing abundant antibody production were boosted by an i.v. injection with antigen and three days later they were used to generate hybridoma clones that secrete monoclonal antibodies.

None of the mice immunized with Aβ vaccines or the anti-Aβ ascites-producing mice displayed ill effects even though some of

those induced antibodies cross-react with mouse Aβ and mouse amyloid precursor protein.

Hybridoma production I. A hybridoma fusion was performed using the spleen of a mouse immunized with the phenylalanine statine transition state Aβ-KLH antigen. Spleen cells from mice with the highest titre were fused with mouse myeloma NS-1 cells to establish hybridomas according to standard procedures (Köhler et al., *Nature* 256:495 (1975); R. H. Kennett, Fusion Protocols. Monoclonal Antibodies, eds. R.H. Kennett, T.J. McKearn and K.B. Bechtol. Plenum Press, New York. 365-367 pp. (1980)).

^{125}I-Aβ binding assay. $A\beta_{1-40}$ and $A\beta_{1-43}$ were radiolabeled with ^{125}I and the iodinated peptide then separated from unlabeled material by HPLC to give quantitative specific activity (~2000 Ci/mmol) (Maggio et al., *Proc. Natl. Acad. Sci.* 89:5462-5466 (1992)). This probe was incubated for 1h at 23^{o}C with either purified anti-Aβ antibodies or media taken from hybridoma clones producing anti-Aβ antibodies. A polyethylene glycol separation method was used to detect the amount of $^{125}I-A\beta_{1-43}$ bound to antibody. By using serial dilution, this assay can provide relative binding affinities for the different hybridoma supernatants or purified antibodies.

Solid phase Aβ proteolytic assay. A solid phase ^{125}I-labeled Aβ assay was developed to screen anti-transition state antibody hybridoma supernatants for specific proteolytic activity. The Cys-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Tyr-amide peptide (SEQ ID NO:5) encompassing amino acids 14-25 of Aβ was radiolabeled with ^{125}I and the iodinated peptide was then separated from unlabeled material by HPLC. The highly radioactive Aβ peptide was coupled to a thiol-reactive, iodoacetyl-Sepharose gel to form an irreversible linkage. Antibodies were added to the labeled Aβ, which was then assayed

for progressive release of soluble ^{125}I-peptide from the solid phase matrix at pH 7, 25°C. This assay was verified by the ability of several different proteases in to rapidly hydrolyze this Sepharose-linked Aβ substrate. Release of soluble ^{125}I-peptide increased with incubation time.

Although Aβ is cleaved by several naturally occurring proteases, preliminary tests indicated that interference from high levels of background hydrolysis was not a problem when assaying hybridoma supernatants of clones that did produce catalytic antibodies. A further precaution that can be taken against exogenous proteases is carrying out all hybridoma cell fusions and cell culturing in serum-free media.

TLC Aβ proteolytic assay. A thin layer chromatography-based autoradiography assay was used to obtain more definitive evidence for antibody-mediated cleavage of Aβ. Selected anti-phenylalanine statine Aβ transition state clones were expanded and ascites production induced. The different monoclonal antibodies were isolated using protein A-Sepharose. The cleavage assay used ^{125}I-A$\beta_{1\text{-}40}$ and an ^{125}I-labeled 17-mer, encompassing amino acids 9-25 of Aβ. Binding of the two ^{125}I-labeled peptides to the purified monoclonal antibodies 5A11 and 6E2 was examined using either a PEG precipitation assay or by a co-electrophoresis method. Peptide cleavage was tested by adding the antibodies to the ^{125}I-peptides, incubating and then spotting the reaction mix onto polyamide thin layer sheets. The chromatographs were developed in different solvents (eg. 0.5 N HCl, 0.5 N NaOH or pH 7 phosphate buffer) and the migration of ^{125}I-products was followed by exposing the sheet using a quantitative phosphoimager system.

Screen and isolate select anti-Aβ antibodies. An ELISA was used to initially screen for anti-Aβ and anti-transition state Aβ peptide monoclonal antibodies. Both the transition state peptide

and the corresponding natural Aβ peptide were adsorbed onto separate microtitre plates. The hybridoma supernatants were screened using two assays so that the relative binding to both native and transition state Aβ peptides could be quantitated. Clones producing monoclonal antibodies that preferentially recognized the transition state or bound Aβ with high affinity were selected for expansion and further study.

Propagation and purification of monoclonal antibodies. Selected clones producing anti-Aβ antibodies and clones producing anti-receptor antibodies were injected into separate pristane-primed mice. Ascites were collected and the specific monoclonal antibodies isolated. Purification of antibodies from ascites was accomplished using a Protein A column or alternatively, antibodies were isolated from ascites fluid by $(NH_4)_2SO_4$ precipitation and passage over an S-300 column to obtain the 150 kDa immunoglobulin fraction. Monovalent Fab fragments were prepared and isolated by established methods. Their purity was evaluated by SDS-PAGE under reducing and non-reducing conditions. 50-100 mg of purified monoclonal antibody was routinely obtained from each ascites-bearing mouse.

Further characterization of catalytic activity on Aβ substrates. To fully define the hydrolytic properties of the isolated anti-transition state antibodies some very important controls can be run. First the ability to completely block catalytic antibody activity with the appropriate transition state peptide can be verified. This non-cleavable "inhibitor" should bind much more tightly to the antibody combining sites and thereby prevent substrate binding or cleavage. Substrate specificity can be further established by showing no cleavage of a sham Aβ peptide having a different amino acid sequence. The products of hydrolysis can also be fully characterized by HPLC, amino acid and mass spectral analysis. Control antibodies that are not

directed against the transition state Aβ can be tested and confirmed to produce no catalysis. Finally, catalytic activity can be shown to reside in the purified Fab fragments of the anti-transition state antibody.

Purified anti-Aβ antibodies dissolve preformed Aβ aggregates. (Walker et al., *Soc. Neurosci. Abstr.* 21:257 (1995), Zlokovic, B.V. *Life Sciences* 59: 1483-1497 (1996)). Aβ precipitates were formed and measured *in vitro* (Yankner et al., *Science* 250: 279-282 (1990), Kowall et al., *Proc. Natl. Acad. Sci.* 88: 7247-7251 (1991)). A radioactive assay was used to quickly screen the different monoclonal antibodies produced for an ability to dissolve preformed Aβ aggregates. After adding ^{125}I-Aβ to unlabeled soluble peptide, aggregates were formed by bringing the solution to pH 5 or by stirring it overnight in PBS. An aliquot of the labeled aggregate was incubated for 1 hr with either PBS, the 5A11 anti-Aβ antibody or an equal amount of an irrelevant mouse antibody (7D3, anti-human transferrin receptor). After centrifugation, the level of radioactivity in the precipitate was measured.

Generation of vectorized anti-Aβ/anti-receptor bispecific antibodies. The anti-Aβ antibodies were chemically coupled to anti-human transferrin receptor and anti-mouse transferrin receptor antibodies by different methods (Raso et al., *J. Biol. Chem.* 272: 27623-27628 (1997); Raso et al., Monoclonal antibodies as cell targeted carriers of covalently and non-covalently attached toxins. *In* Receptor mediated targeting of drugs, vol. 82. G. Gregoriadis, G. Post, J. Senior and A. Trouet, editors. NATO Advanced Studies Inst., New York. 119-138 (1984)). A rapid thioether linkage technique was used to form strictly bispecific hybrids using Traut's reagent and the heterobifunctional SMBP reagent. One component was sparingly substituted with thiol groups (SH). These readily reacted to form a thioether linkage

upon mixture with the maleimido-substituted (M) second component following the reaction:

$$Ab_A\text{-SH} + Ab_B\text{-M} \rightarrow Ab_A\text{-S-}Ab_B$$

Gel filtration of the reaction mixture on an S-300 column yielded the purified dimer which was 300 kDa and had two sites for binding Aβ plus two sites for attachment to transferrin receptors on brain capillary endothelial cells. Non-targeted control hybrids were formed by linking a nonspecific MOPC antibody to the anti-Aβ antibody. This hybrid antibody does bind Aβ, but, being non-reactive with transferrin receptors, should not cross the blood-brain barrier.

$F(ab')_2$ fragments of the two different antibody types can similarly be thioether-linked to form Fc-devoid reagents that cannot bind complement which might otherwise cause neurotoxic effects. These smaller bispecific hybrids (100 kDa) can be formed by reducing the intrinsic disulfides which link the heavy chains of $F(ab')_2$ fragments (Raso et al., *J. Immunol.* 125:2610-2616 (1980)). The thiols generated are stabilized and Ellman's reagent (E) is used to activate these groups on one of the components (Brennan et al., *Science* 229: 81-83 (1985)). Exclusively bispecific $F(ab')_2$ hybrids can be formed upon mixing the reduced Fab' with an activated Fab' having the alternate specificity according to the reaction:

$$Fab'_A\text{-SH} + Fab'_B\text{-SS-E} \rightarrow Fab'_A\text{-SS-}Fab'_B + \text{E-SH}$$

Purification on an S-200 column will isolate hybrids with one site for binding Aβ and one site for interaction with the target epitope on the brain capillary endothelial cells.

A similar approach can be used to make even smaller disulfide-linked single chain Fv heterobispecific dimers, Fv_A-SS-Fv_B (50 kDa), to cross the blood-brain barrier. Soluble Fvs can

be constructed to possess a carboxyl-terminal cysteine to facilitate the disulfide exchange shown in the reaction below, and create 50 kDa heterodimers exclusively:

$$Fv_A\text{-}SH + Fv_B\text{-}SS\text{-}E \rightarrow Fv_A\text{-}SS\text{-}Fv_B + E\text{-}SH$$

In side by side comparisons between whole antibody and either Fab' or Fv based bispecific reagents, the latter have proven to be moderately more effective on a molar basis for cell uptake via the transferrin receptor-mediated pathway (Raso et al., J. Biol. Chem. 272: 27623-27628 (1997)). Since these smaller constructs are monovalent for the cell-surface epitope, those findings dispel the notion that cross-linking of two surface receptors is necessary for the cellular uptake of immunocomplexes.

Functional assays for dual binding activity of bispecific antibodies. The capacity of the hybrid reagent to bind ^{125}I-Aβ was compared with that of the parent anti-Aβ antibody in a standard PEG binding assay (see Table 8 for binding assays).

The ability of the appropriate bispecific antibodies to attach to transferrin receptor bearing human or mouse cells was confirmed by cytofluorimetry. The bispecific antibody was reacted with transferrin receptor positive human or mouse cells and probed using either a rat IgG-specific or mouse IgG-specific fluorescent secondary antibody reagent.

Measurement of Aβ binding using ^{125}I-Aβ and a polyethylene glycol separation. To ensure bispecificity, hybrid reagents were tested for a capacity to mediate the attachment of ^{125}I-Aβ to receptor-bearing cells. Transferrin receptor positive cells were treated with the hybrid reagent, washed away unbound material and then exposed these cells to ^{125}I-A$\beta_{1\text{-}40}$. The cells were washed and the amount of cell-bound radioactivity was compared to control cells

which had been identically prepared except that pretreatment with bispecific antibody was omitted.

Capillary depletion. The bispecific antibody was labeled with ^{125}I and injected i.v. into normal mice. After different lengths of time the mice were sacrificed and the amount of ^{125}I-bispecific antibody that crossed the blood-brain barrier and entered the brain was gauged by a mouse capillary depletion method (Friden et al., *J. Pharm. Exper. Ther.* 278:1491-1498 (1996); Triguero et al., *J. Neurochem.* 54: 1882-1888 (1990)). The amount of vectorized bispecific antibody found in the brain parenchyma or brain capillary fractions was measured following differential density centrifugation of the brain homogenate. These values were plotted as a function of time after i.v. injection. Progressive passage from capillaries into the parenchyma indicates active transcytosis across the blood-brain barrier.

Immunoscintigraphy. A non-invasive method for monitoring intracerebral delivery process which involves visualizing the entry of a radiolabeled bispecific antibody into the brain of live mice, can also be used. Radiolabeled vectorized bispecific antibody (^{125}I-R17/5A11) or a non-vectorized control bispecific antibody were administered to separate mice. Sequential brain images were accumulated at 1, 6, 24 and 48 hours following i.v. administration of the ^{125}I-labeled bispecific antibody probes. The animals were chemically immobilized during exposure using ketamine/xylazine anesthesia. This imaging technology could be very useful for determining if circulating anti-Aβ antibodies will prevent i.v. administered ^{125}I-Aβ from entering the brain. Digital scintigraphy data was quantified using standards and the integration functions provided in the analysis software.

CLAIMS

is characterized by the ability to

1. An antibody which ~~catalyzes~~ hydrolysis of β-amyloid at a predetermined amide linkage.

2. The antibody of Claim 1 which catalyzes hydrolysis of the amide linkage between residues $\overset{39}{X}$ and $\overset{40}{Y}$ of β-amyloid.

[VR: THE APPROPRIATE RESIDUES WILL BE FILLED IN FOR EACH OF THE DIFFERENT CATALYTIC ANTIBODIES]

40-41 41-42 19-20 20-21 38-39

3. The antibody of Claim 1 which preferentially binds a transition state analog which mimics the transition state adopted by β-amyloid during hydrolysis at a predetermined amide linkage, and also binds to natural β-amyloid with sufficient affinity to detect using an ELISA.

4. The antibody of Claim 1 which preferentially binds a transition state analog which mimics the transition state adopted by β-amyloid during hydrolysis at a predetermined amide linkage, and does not bind natural β-amyloid with sufficient affinity to detect using an ELISA.

5. A vectorized antibody which is characterized by the ability to cross the blood brain barrier and the ability to catalyze the hydrolysis of β-amyloid at a predetermined amide linkage.

6. The vectorized antibody of Claim 5 which is a bispecific antibody..

7. The vectorized antibody of Claim 6 which has a first specificity for the transferrin receptor and a second specificity for a transition state adopted by β-amyloid during hydrolysis.

8. The vectorized antibody of Claim 7 which catalyzes hydrolysis of β-amyloid between residues X and Y.

[VR: THE APPROPRIATE RESIDUES WILL BE FILLED IN FOR EACH OF THE DIFFERENT CATALYTIC ANTIBODIES]

9. A method for sequestering free β-amyloid in the bloodstream of an animal, comprising the steps:
   a) providing antibodies specific for β-amyloid; and
   b) intravenously administering the antibodies to the animal in an amount sufficient to increase retention of β-amyloid in the circulation.

10. A method for sequestering free β-amyloid in the bloodstream of an animal, comprising the steps:
   a) providing an antigen comprised of an epitope which is present on β-amyloid endogenous to the animal; and
   b) immunizing the animal with the antigen of step a) under conditions appropriate for the generation of antibodies which bind endogenous β-amyloid.

11. A method for reducing levels of β-amyloid in the brain of an animal, comprising the steps:
   a) providing antibodies specific for β-amyloid endogenous to the animal; and
   b) intravenously administering the antibodies to the animal in an amount sufficient to increase retention of β-amyloid in the circulation of the animal.

12. The method of Claim 11 wherein the antibodies specific for β-amyloid are catalytic antibodies which catalyze hydrolysis of β-amyloid at a predetermined amide linkage.

13. The method of Claim 11 wherein the antibodies are monoclonal.

14. The method of Claim 11 wherein the antibodies are polyclonal.

15. The method of Claim 11 wherein the antibodies specifically recognize epitopes on the C-terminus of β-amyloid$_{1-43}$.

16. A method for reducing levels of β-amyloid in the brain of an animal, comprising the steps:
    a) providing an antigen comprised of an epitope which is present on β-amyloid endogenous to the animal; and
    b) immunizing the animal with the antigen of step a) under conditions appropriate for the generation of antibodies which bind endogenous β-amyloid.

17. The method of Claim 16 wherein the antigen is a transition state analog which mimics the transition state adopted by β-amyloid during hydrolysis at a predetermined amide linkage.

18. The method of Claim 16 wherein the antigen is ....

19. The method of Claim 17 wherein the antibodies generated have a higher affinity for the transition state analog than for natural β-amyloid.

20. The method of Claim 17 wherein the antibodies generated catalyze hydrolysis of endogenous β-amyloid.

21. A method for preventing the formation of amyloid plaques in the brain of an animal, comprising the steps:
    a) providing an antigen comprised of an epitope which is present on β-amyloid endogenous to the animal; and
    b) immunizing the animal with the antigen of step a) under conditions appropriate for the generation of antibodies which bind endogenous β-amyloid.

22. The method of Claim 21 wherein the antigen is a transition state analog which mimics the transition state adopted by β-amyloid during hydrolysis at a predetermined amide linkage.

23. A method for reducing levels of circulating β-amyloid in an animal, comprising the steps:
    a) providing an antigen comprised of an epitope which is a mimic of a predetermined hydrolysis transition state of a β-amyloid polypeptide endogenous to the animal; and
    b) immunizing the animal with the antigen of step a) under conditions appropriate for the generation of antibodies to the β-amyloid hydrolysis transition state.

24. A method for reducing levels of circulating β-amyloid in an animal, comprising the steps:
    a) providing antibodies which catalyze the hydrolysis of β-amyloid endogenous to the animal; and
    b) intravenously administering the antibodies to the animal.

25. A method for preventing the formation of amyloid plaques in the brain of an animal, comprising the steps:
    a) providing antibodies which catalyze hydrolysis of β-amyloid produced by the animal at a predetermined amide linkage; and
    b) administering the antibodies to the animal in an amount sufficient to cause a significant reduction in β-amyloid levels in the blood of the animal.

26. A method for reducing levels of β-amyloid in the brain of an animal, comprising the steps:
    a) providing vectorized bispecific antibodies competent to transcytose across the blood brain barrier, which catalyze hydrolysis of β-amyloid of the animal at a

predetermined amide linkage; and

b) intravenously administering the antibodies to the animal.

27. The method of Claim 26 wherein the vectorized bispecific antibodies specifically bind the transferrin receptor.

28. The method of Claim 26 wherein the vectorized bispecific antibodies catalyze hydrolysis of the amide linkage between residues X and Y of β-amyloid.

[VR: THE APPROPRIATE RESIDUES WILL BE FILLED IN FOR EACH OF THE DIFFERENT CATALYTIC ANTIBODIES]

29. A method for disaggregating amyloid plaques present in the brain of an animal comprising the steps:

a) providing vectorized bispecific antibodies competent to transcytose across the blood brain barrier, which catalyze hydrolysis of β-amyloid produced by the animal at a predetermined amide linkage; and

b) intravenously administering the antibodies to the animal in an amount sufficient to cause significant reduction in β-amyloid levels in the brain of the animal.

30. A method for disaggregating amyloid plaques present in the brain of an animal, comprising the steps:

a) providing antibodies which catalyze hydrolysis of β-amyloid produced by the animal at a predetermined amide linkage; and

b) administering the antibodies to the animal.

31. The method of Claim 30 wherein the antibodies are bispecific vectorized antibodies competent for transcytosis across the blood-brain barrier.

32. A method for generating antibodies which catalyze hydrolysis of a protein or polypeptide comprising the steps:
   a) providing an antigen, the antigen being comprised of an epitope which has a statine analog which mimics the conformation of a predetermined hydrolysis transition state of the polypeptide;
   b) immunizing an animal with the antigen under conditions appropriate for the generation of antibodies to the hydrolysis transition state.

33. The method of Claim 32 wherein the protein is β-amyloid.

34. A method for generating antibodies which catalyze hydrolysis of a protein or polypeptide comprising the steps:
   a) providing an antigen, the antigen being comprised of an epitope which has a reduced peptide bond analog which mimics the conformation of a predetermined hydrolysis transition state of the polypeptide;
   b) immunizing an animal with the antigen under conditions appropriate for the generation of antibodies to the hydrolysis transition state.

35. The method of Claim 34 wherein the protein is β-amyloid.

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May 25, 1999

Dr. Victor Raso, Ph.D.
Boston Biomedical Research Institute
20 Staniford St.
Boston, MA 02114

Re: BBRI-2004
Immunological Control of Beta-Amyloid Levels In Vivo

Dear Dr. Raso:

Enclosed please find a draft patent application, for your review. This revision differs from the draft transmitted to you on May 13, 1999. Please review the draft for accuracy and completeness. Note that there are a few questions addressed to you within the body of the text. We will incorporate any revisions indicated and file the application in short order (within hours of receipt of your comments).

Best regards,

Shayne Huff

Shayne Huff

cc: Ms. Pamela Torpey, w/enc.
Kevin Farrell, Esqire, w/o enc.

BBRI\CURR\2004.L1

DRAFT

IMMUNOLOGICAL CONTROL OF BETA-AMYLOID LEVELS IN VIVO

Background of the Invention

Alzheimer's disease is a progressive and ultimately fatal form of dementia that affects a substantial portion of the elderly population. Definitive diagnosis at autopsy relies on the presence of neuropathological brain lesions marked by a high density of senile plaques. These extracellular deposits are found in the neo-cortex, hippocampus and amygdala as well as in the walls of the meningeal and cerebral blood vessels. The principal component of these plaques is a 39 to 43 residue β-amyloid peptide. Each plaque contains approximately 20 fmole (80 picograms) of this 4 kDa peptide (Selkoe et al., *J. of Neurochemistry 46*: 1820 (1986)). Apolipoprotein E and neurofibrillary tangles formed by the microtubule-associated tau protein are also often associated with Alzheimer's disease.

β-amyloid is proteolytically cleaved from an integral membrane protein called the β-amyloid precursor protein. The gene which codes for this protein in humans is found on chromosome 21 (St George-Hyslop et al., *Science 235*: 885 (1987), Kang et al., *Nature* 325: 733 (1987)). Numerous cultured cells and tissues (eg. brain, heart, spleen, kidney and muscle) express this β-amyloid precursor protein and also secrete the 4 kDa β-amyloid fragment into culture media, apparently as part of a normal processing pathway.

While it is difficult to establish an absolute causal relationship between β-amyloid or the plaques it forms and Alzheimer's disease, there is ample evidence to support the pathogenic role of β-amyloid. For example, patients with Down's syndrome have an extra copy of the β-amyloid precursor protein gene due to trisomy of chromosome 21 (St George-Hyslop et al., *Science 235*: 885 (1987), Kang et al., *Nature* 325: 733 (1987)). They correspondingly develop an early-onset Alzheimer's disease neuropathology at 30-40 years of age. Moreover, early-onset familial Alzheimer's disease can result from mutations in the β-amyloid precursor protein gene which fall within or adjacent to the β-amyloid sequence (Hardy, J., *Nature Genetics 1*: 233 (1992)). These observations are consistent with the notion that

deposition of β-amyloid as plaques in the brain are accelerated by an elevation in its extracellular concentration (Scheuner et al., *Nature Med. 2:* 864 (1996)). The finding that β-amyloid is directly neurotoxic both *in vitro* and *in vivo* (Kowall et al. *Proc. Natl. Acad. Sci. 88*: 7247 (1991)), ~~opens the possibility~~ suggest that soluble aggregated β-amyloid, not the plaques per se, may produce the pathology.

Observations have indicated that amyloid plaque formation may proceed by a crystallization type mechanism (Jarrett et al., *Cell 73*: 1055 (1993)). According to this model, the seed that initiates plaque nucleation is an β-amyloid which is 42 or 43 amino acids long ($A\beta_{1-43}$). The rate-determining nucleus formed by $A\beta_{1-43}$ or $A\beta_{1-42}$ allows peptides $A\beta_{1-40}$ or shorter to contribute to the rapid growth of an amyloid deposit. This nucleation phenomenon was demonstrated *in vitro* by the ability of $A\beta_{1-42}$ to cause the instantaneous aggregation of a kinetically stable, supersaturated solution of $A\beta_{1-40}$. That finding has led to the possibility that $A\beta_{1-40}$ might be relatively harmless in the absence of the nucleation peptides $A\beta_{1-42}$ or $A\beta_{1-43}$. Indeed, elevated levels of these long peptides have been found in the blood of patients with familial Alzheimer's disease (Scheuner et al., *Nature Med. 2:* 864 (1996)). Moreover, $A\beta_{1-42}$ or $A\beta_{1-43}$ was found to be the predominant form deposited in the brain plaques of many Alzheimer's disease patients (Gravina et al., *J. of Biol. Chem. 270*: 7013 (1995)).

Given the central role played by β-amyloid, it has become increasingly important to understand the interrelationship between the different pools of these molecules in the body. Free β-amyloid present in the blood most likely arises from peptide released by proteolytic cleavage of β-amyloid precursor protein present on cells in the peripheral tissues. Likewise most of the free β-amyloid found in the brain and cerebrospinal fluid is probably derived from peptide released by secretase cleavage of β-amyloid precursor protein expressed on brain cells. The

peptides are identical regardless of origin, and the results from several studies suggest an intercommunication between these pools.

Brief Description of the Figures

Figure 1 is an amino acid sequence listing (SEQ ID NO: 1) of the 43 residue β-amyloid peptide ($A\beta$).

Figure 2 is an amino acid sequence listing (SEQ ID NO: 2) of the antigenic peptide made from the N-terminal sequence of ~~$A\beta$~~ β-amyloid ($A\beta_{1-16}$).

Figure 3 is an amino acid sequence listing (SEQ ID NO: 3) of the antigenic peptide made from the central region of ~~$A\beta$~~ β-amyloid ($A\beta_{10-25}$).

Figure 4 is an amino acid sequence listing (SEQ ID NO: 4) ($A\beta_{35-43}$) of the antigenic peptide made from the C-terminal sequence of ~~$A\beta$~~. β-amyloid

Figure 5 is a diagrammatic representation of data from an ELISA comparing monoclonal antibody binding to $A\beta_{35-43}$ and $A\beta_{1-43}$ versus $A\beta_{1-40}$.

Figure 6 indicates the amide linkages in the peptide made from the $A\beta$-amyloid C-terminal sequence (SEQ ID NO: 4) that were independently replaced with a statyl moiety, to generate the different statine transition state analogs of the peptide.

Figure 7 indicates the amide linkages in the peptide made from the $A\beta$-amyloid central sequence (SEQ ID NO: 3) that were independently replaced with a statyl moiety, to generate the different phenylalanine statine transition state analogs of the peptide.

Figure 8 is a structural comparison between the native ~~$A\beta$~~ β-amyloid peptide and the transition state phenylalanine statine ~~$A\beta$~~ amyloid peptide analog.

Figure 9 is a structural comparison between the native ~~$A\beta$~~ β-amyloid peptide and the reduced peptide bond transition state ~~$A\beta$~~ amyloid peptide analog.

Figure 10 is a formulaic representation of the native C-terminal region of phosphonamidate transition state analog of β-amyloid, and the

the C-terminal region of ~~Aβ~~ β-amyloid ($A\beta_{35-43}$).

Figure 11 indicates the putative transition state for peptide hydrolysis by zinc peptidases, ~~and~~ compared to the phosphonate and phosphonamidate mimics.

Figure 12 is a structural comparison of the native ~~Aβ~~ β-amyloid peptide and the transition state phosphonamidate ~~Aβ~~ β-amyloid peptide which has the peptide link between Gly 38 and Val 39 replaced with a phosphonamidate bond.

Figure 13 is a diagrammatic representation of data from an ELISA which assess the binding of monoclonal antibodies, generated to transition state ~~Aβ~~ β-amyloid peptide analogs, to the normal $A\beta_{1-43}$ and to the phenylalanine statine transition state ~~Aβ~~ β-amyloid peptide.

Figure 14 is a diagrammatic representation of data from an ELISA comparing antibody binding to the statine transition state ~~Aβ~~ β-amyloid peptide versus native $A\beta_{1-43}$. and native $A\beta_{1-40}$

Figure 15 is a graph of data showing the cleavage of 125I-Aβ-sepharose by monoclonal antibodies generated to transition state analogs of ~~Aβ~~ β-amyloid.

Figure 16 is a diagrammatic representation of data which quantitates ~~indicates~~ the attachment of bispecific antibody to receptor-positive cells.

Figure 17 is a diagrammatic representation of data obtained from experiments ~~obtained~~ ~~following~~ designed to track ~~indicating~~ the transcytosis of vectorized bispecific antibody into brain.

Detailed Description of the Invention

The present invention relates to immunologically based methods for controlling levels of β-amyloid in the body of an animal. The invention is based on the finding that antibodies specific for β-amyloid are able to bind β-amyloid in the presence of a physiological level of human serum albumin. The invention is also based on the finding that an animal can tolerate the presence of antibodies specific for β-amyloid in amounts sufficient to sequester β-amyloid in the bloodstream.

One aspect of the present invention related to a method for sequestering free β-amyloid in the bloodstream of an animal. The soluble and insoluble forms of β-amyloid present within an animal are in dynamic equilibrium. Soluble β-amyloid is thought to translocate between blood and cerebrospinal fluid. Insoluble β-amyloid aggregates deposit from the soluble pool in the brain, as amyloid plaques. Results detailed in the Exemplification section below indicate that intravenous administration of antibodies specific for β-amyloid to an animal impedes the passage of soluble β-amyloid out of the peripheral circulation. This occurs because the β-amyloid specific antibodies, which are restricted to the peripheral circulation, bind to β-amyloid and sequester it in the circulation. Such sequestration is accomplished through intravenous administration of an appropriate amount of antibodies specific for β-amyloid to the animal. The amount of antibody ~~administered~~ which is sufficient to produce sequestration when administered is dependent upon various factors (e.g. specific characteristics of the antibody to be delivered, the size, metabolism, and overall health of the animal) and ~~should~~ are to be determined on a case by case basis.

Administered antibodies can be monoclonal antibodies, a mixture of different monoclonal antibodies, polyclonal antibodies, or any combination therein. In one embodiment, the antibodies bind to the C-terminal region of β-amyloid. Such antibodies specifically bind the less abundant, but more noxious $A\beta_{1-43}$ species in the blood as opposed to the smaller and less detrimental $A\beta_{1-40}$. In another embodiment, a combination of antibodies having specificity for various regions of β-amyloid are administered. In another embodiment, antibodies which catalyze the hydrolysis of β-amyloid, discussed in more detail below, are administered either alone or in combination with other anti-β-amyloid antibodies.

The animal to which ~~is administered~~ the antibodies are administered is any animal which has circulating soluble β-amyloid. In one embodiement, the animal is a human. The human

may be a healthy individual, or alternatively, may be suffering from or at risk for a disease in which elevated β-amyloid levels are thought to play a role, for example a neurodegenerative disease such as Alzheimer's disease.

A related aspect of the present invention is a method for sequestering free β-amyloid in the bloodstream of an animal by ~~generating~~ stimulating an ~~immune resonse~~ ~~in~~ within the animal ~~to~~ endogenous β-amyloid. The results detailed in the Exemplification below indicate that an animal can tolerate the induction of an immune response which produces antibodies to endogenous β-amyloid, and that the presence of such antibodies will alter the distribution of β-amyloid in the body, in a similar manner as the above described method of administering β-amyloid binding antibodies. The immune response to endogenous β-amyloid is generated by immunizing the animal with one or more antigens comprised of epitopes present on the endogenous β-amyloid ~~endogenous to the animal~~. Epitopes present on the innoculated antigens can correspond to epitopes present on any region of the β-amyloid molecule. In a preferred embodiment, epitopes found on the C-terminal region of β-amyloid are used to generate antibodies which specifically bind the $A\beta_{1-43}$ species as opposed to the smaller $A\beta_{1-40}$. In an alternate embodiment, a combination of different epitopes ~~and antigen-containing epitopes~~ are administered to generate a variety of antibodies to β-amyloid. A more generalized immune response is generated by immunizing either with a mixture of different small peptide antigens or with the full-length 43 residue β-amyloid peptide. In another embodiment, antigens used for inoculation include transition state analogs of β-amyloid peptides to induce antibodies which have catalytic activity directed towards β-amyloid hydrolysis, described in detail below.

The immunoreactivity of the antigens can be enhanced by a variety of methods, many of which involve coupling the antigen to an immunogenic carrier. In addition, various methods are known and available to one of skill in the art for specifically

enhancing the immunogenicity of endogenous molecules or the epitopes contained therein. Various ~~M~~modifications can be made to the β-amyloid antigen(s) described herein to render it more compatible for human use. For example, the peptide(s), can be genetically engineered into appropriate antigenic carriers, or DNA vaccines can be designed.

The above techniques for sequestering β-amyloid in the circulation are also useful for reducing the levels of β-amyloid in the brain ~~brain β-amyloid levels~~. Because the formation of amyloid plaques in the brain is dependent, at least in part, on the levels of free β-amyloid present in the brain, reducing brain β-amyloid levels of an animal will in turn, reduce the formation of amyloid plaques in the brain. Therefore, the above techniques are ~~also~~ useful for preventing the formation of amyloid plaques in the brain of an animal. This is especially applicable to an animal which is considered at risk for the development of amyloid plaques; a risk which may result from a genetic predisposition or from environmental factors. Administration of antibodies, or immunization of the animal to produce endogenous antibodies, ~~for~~ to β-amyloid can be of therapeutic benefit to such an animal (e.g. a human who has a family history of Alzheimer's disease, or who is diagnosed with the disease).

Another aspect of the present invention relates to antibodies which are characterized by the ability to catalyze the hydrolysis of β-amyloid at a predetermined amide linkage. Experiments detailed in the Exemplification section demonstrate the generation of different antibodies which have proteolytic activity towards β-amyloid. Such antibodies are generated by immunizing an animal with a~~n~~ antigen which is a ~~β-amyloid peptide~~ antigen which is a ~~β analog~~ stable transition state (analog) of the β-amyloid peptide. ~~analog.~~ A transition state analog mimics the ~~transition~~ state of the hydrolysis that β-amyloid adopts during hydrolysis ~~at~~ of a specific ~~predetermined~~ amide linkage. Transition state analogs useful for generating the catalytic antibodies include, without limitation, statine, phenylalanine statine, phosphonate, phosphonamidate, and

of an amide linkage within the β-amyloid peptide.

reduced peptide bond transition state analogs.

Antibodies generated to epitopes unique to the transition state preferentially bind β-amyloid in the transition state. Binding of these antibodies stabilizes the transition state, which leads to hydrolysis of the corresponding amide bond. The particular amide linkage to be hydrolyzed is chosen based upon the desired cleavage product. For example, cleavage of full length β-amyloid into two peptide fragments which cannot aggregate into amyloid plaques would be of therapeutic use in the methods disclosed herein. Monoclonal antibodies which recognize the transition state of specific amide likages in β-amyloid include ..... **[VR: PLEASE PROVIDE LIST OF AMIDE LINKAGES THAT THE MONOCLONAL ANTIBODIES RECOGNIZE.]**

At least two different classes of antibodies are generated by the above methods. The first class preferentially binds the transition state analog, and also detectably cross reacts with natural β-amyloid, when using ~~the~~ an ELISA ~~detailed in~~ (see the Exemplification ~~section~~, to detect binding. The second class binds the transition state analog, and does not ~~detectably~~ cross react to natural β-amyloid, at levels which are detectable via ~~using the~~ ELISA (see the procedure detailed in the Exemplification section ~~to detect binding~~ for specific ELISA procedures). Both classes of antibodies have potential value as catalytic antibodies. These ~~respective~~ binding characteristics ~~affinities of an anti-transition state antibody~~ are ~~is~~ likely to reflect ~~its~~ the characteristics of native vs. transition state activity ~~at catalyzing hydrolysis~~ of the antibody. It is thought that in order for an antibody to ~~have activity at~~ catlyze ~~ing~~ hydrolysis of a protein, it must posess at least a minimal ability to bind the natural (non-transition) state of the protein. Antibodies which retain significant binding for β-amyloid, (e.g. antibodies that strongly cross react with natural β-amyloid) may be more efficient at catalyzing hydrolysis due to a higher efficiency of binding the β-amyloid. Once bound, these antibodies force the protein into a transition state conformation for hydrolytic cleavage. Alternatively, antibodies which only minimally cross react with natural β-amyloid, although less

catalysis activity of the antibody.

sp?

efficient at binding native β-amyloid, are likely to be more efficient at forcing the bound β-amyloid into the transition state conformation for hydrolytic cleavage. It should be pointed out that failure to detect binding of the anti-transition state antibodies to natural β-amyloid by the ELISA methods presented in the Exemplification herein does not necessarily reflect an inability to bind natural β-amyloid sufficiently to function as a catalytic antibody. More likely, a lack of detection merely reflects the sensitivity limitations of the assay.

~~Note~~ Antibodies ~~with~~ which have substantial affinity for the predicted cleavage products of the native β-amyloid peptide may be subject to product inhibition and might therefore exhibit low turnover. Such undesireable antibodies can be identified by secondary screening using peptides which contain epitopes of the predicted cleavage products (e.g. via ELISA).

In a preferred embodiment, the antibodies are monoclonal. monoclonal antibodies ~~These~~ ~~can be~~ are produced by immunizing an animal (e.g. mouse, guinea pig, or rat) with the transition state analog antigen, and subsequently producing hybridomas from the animal, by standard procedures. Hybridomas which ~~producing~~ produce the desired monoclonal antibodies are then identified by screening. One example of a screening method is presented in the Exemplification section which follows. In another embodiment, the antibodies are polyclonal. Polyclonal antibodies are generated by immunizing an animal (e.g. a rabbit, chicken, or goat) with antigen and obtaining sera from the animal. Polyclonal antibodies ~~with~~ which have the desired binding specificities can be further purified from the sera by one of skill in the art through the course of routine experimentation.

Another aspect of the present invention is the use of statine and reduced peptide bond analogs to elicit catalytic antibodies having proteolytic activity. The Exemplification section below details methods for using statine analogs as antigen in the production of catalytic antibodies, and also lists examples of anti-transition-state antibodies generated using these methods. The "statyl" moiety is derived from naturally

evolved protease transition state inhibitors like amastatin, pepstatin, and bestatin. These naturally-occurring statine-based inhibitors have been used to effectively block the activity of aminopeptidases, aspartic proteases and the HIV protease. Synthetic peptides containing a statine residue offer novel features for the induction of catalytic antibodies. The statyl moiety has a tetrahedral bond geometry, its length is extended by two CH_2 units, it has a strategically placed OH group and the structure has no charge. The presence of the additional CH_2 units is expected to elicit a more elongated antibody combining site, and antibodies generated to this site will induce extra strain on the peptide substrate, producing an accellerated catalysis. In addition, the OH group in these statine analogs is thought to better approximate the position and chemistry of the true transition state. Statine-based transition-state analogs should therefore elicit a class of antibodies which is significantly different from those obtained from the more commonly used negatively charged phosphonate analogs.

Reduced peptide bond analogs introduce a tetrahedryl configuration, without increasing the distance between amino acid residues. This feature should more closely approximate the true transition state geometry, than previously used analogs. A positively charged secondary amine replaces the amide nitrogen of the natural polypeptide and should elicit a complementary negatively charged side chain at a proximal locus in the antibody combining site. The presence of Such ancillary glutamyl or aspartyl groups ~~present~~ on the antibody ~~could~~ will assist antibody-mediated catalysis of peptide cleavage via acid-base exchange. Reduced peptide bond-based transition-state analogs should therefore elicit a class of antibodies which is significantly different from those obtained from using the more commonly used negatively charged phosphonate analogs. Both reduced peptide bond analogs and ~~also~~ statine analogs can be used to produce a wide variety of specific transition state analog antigens ~~which mimic~~ the ~~a wide variety of~~ proteins ~~or~~ for a polypeptides. These antigens can in turn be used to generate the respective catalytic antibodies ~~to a wide var~~

Administration of the β-amyloid catalytic antibodies described above finds use in the above described methods for 1) sequestering free β-amyloid in the bloodstream of an animal, 2) reducing levels of β-amyloid in the brain of an animal, and 3) preventing the formation of amyloid plaques in the brain of an animal. Experiments presented in the Exemplification demonstrate that immunization of an animal with a transition state analog results in the generation of an immune response to produce antibodies which recognize the transition state, and which catalyze hydrolysis of the β-amyloid protein. This indicates that the transition state analogs can be used as antigens in these methods to induce the production of antibodies in the animal which recognize and catalyze cleavage of endogenous β-amyloid.

Methods which involve reducing overall levels of β-amyloid in an animal through the proteolytic action of the above described catalytic antibodies are also encompassed by the present invention. The presence of functional catalytic antibodies in the circulation of an animal reduces the level of β-amyloid in the circulation. Accordingly, the present invention provides a method for reducing levels of circulating β-amyloid in an animal by introducing the above described catalytic antibodies into the animal.

The present invention also provides a method for reducing levels of circulating β-amyloid in an animal by immunizing the animal with a β-amyloid transition state analog to induce antibody production. The use and design of such vaccines is described above, and ~~vaccines~~ are detailed in the Exemplification section below. Administration of the antibodies to the animal is preferably via intravenous administration. Such antibodies are either monoclonal, mixed monoclonal, polyclonal or any mixture thereof. The origin of the antibody may affect the half-life of the antibody in the animal; antibodies from less related species are more likely to be recognized as foreign by the animal's immune system. Preferably, administered antibodies are derived from a species closely related to the animal, to maximize half-

life and minimize adverse reactions by the host. Administration of isolated variable region antibody fragments may produce beneficial results in this regard.

The reduction of β-amyloid levels in the circulation of an animal is expected to displace the equilibrium of β-amyloid in the body, and lead to a reduction in the levels of β-amyloid in the brain of the animal through mass action. In this respect, the present invention provides methods for reducing the levels of β-amyloid in the brain of an animal, by either administering catalytic antibodies to the animal, or by administering a transition state analog to induce endogenous antibody production. It follows that these procedures also have value as methods for preventing the formation of amyloid plaques in the brain of an animal, since the resulting reduction in the levels of β-amyloid in the brain of an animal should prevent the formation of amyloid plaques. These procedures also have value as methods for disaggregating amyloid plaques present in the brain of an animal, since evidence indicates that lower brain β-amyloid levels can lead to the disaggregation of plaques.

Another aspect of ~~T~~the present invention ~~also~~ provides ~~for~~ a more direct method~~s~~ of altering the distribution of β-amyloid in the brain by actually delivering anti-β-amyloid antibodies to the brain. Methods described above for reducing levels of β-amyloid in the brain and for preventing aggregation of amyloid plaques depend upon exchange between β-amyloid pools in the circulation the cerebrospinal fluid, the exchange being driven by a disruption of the equilibrium between the pools. In contrast, delivery of anti-β-amyloid antibodies to the brain will directly affect β-amyloid aggregation. Evidence presented in the Exemplification section below indicates that the binding of certain anti-β-amyloid antibodies inhibits the initial aggregation of β-amyloid *in vitro*, and also disaggregates preformed *in vitro* β-amyloid complexes. Moreover, if insoluble peptide is in equilibrium with a low level of soluble β-amyloid, then an anti-β-amyloid binding

antibody could upset this balance and gradually dissolve the precipitate. These observations indicate that the presence of β-amyloid antibodies in the brain will directly inhibit the formation of amyloid plaques and will also disaggregate preformed plaques by disrupting the dynamic equilibrium between soluble β-amyloid and fibrillar β-amyloid deposited as plaques. Furthermore, a highly active catalytic antibody is expected to destroy insoluble β-amyloid plaques by hydrolytically cleaving the constituent aggregated peptides.

One way of delivering antibodies to the brain is by producing vectorized antibodies competent for transcytosis across the blood-brain barrier. Vectorized antibodies are produced by covalently linking an antibody to an agent which promotes delivery from the circulation to a predetermined destination in the body. Examples of such vectorized antibodies can be found in the prior art .... **[VR: PLEASE PROVIDE REFERENCES]** One such agent is another antibody which is directed towards a cell surface component, such as a receptor, which is transported away from the cell surface. Examples of antibodies which confer the ability to trancytose the blood-brain barrier include, without limitation, anti-insulin receptor antibodies, and also anti-transferrin receptors (Saito et al., *Proc Natl Acad Sci USA 92*: 10227-31 (1995); Pardridge et al., *The Primate 12*: 807-816 (1995); **[VR: PLEASE VERIFY THE ACCURACY OF THIS LAST REFERENCE AS IT WAS NOT CLEAR IN THE MATERIALS PROVIDED]** Broadwell et al., Exp Neurol 142: 47-65 (1996)). This first antibody is covalently linked to an antibody which binds β-amyloid. Alternatively, coupling the β-amyloid antibodies to ligands which bind these receptors, e.g. insulin, transferrin, or LDL **[VR: PLEASE PROVIDE COMPLETE NAME OF LDL]** will also produce a vectorized antibody competent for delivery to the brain from the circulation (Descamps et al., *Am. J. Physiol. 270*: H1149-H1158 (1996); Duffy et al., *Brain Res. 420*: 32-38 (1987); Dehouck et al., *J. Cell Biol. 138*: 877-889 (1997)).

A vector moiety can be chemically or genetically attached to

the anti-β-amyloid antibody to facilitate its delivery into the central nervous system. This vector component can be for example, an anti-transferrin receptor or anti-insulin receptor antibody ~~that~~ binds ~~to those~~ receptors on the brain capillary endothelial cells (Bickel et al., *Proc Natl Acad Sci U S A* 90: 2618-22 (1993); Pardridge et al., *J Pharmacol Exp Ther 259*: 66-70 (1991); Saito et al. *Proc Natl Acad Sci U S A* 92: 10227-31(1995); Friden et al., *J. Pharm. Exper. Ther.* 278: 1491-1498 (1996)) which make up the blood-brain barrier. The ~~resulting~~ bifunctional antibody (Raso et al., *J. Biol. Chem.* 272: 27623-27628 (1997); Raso et al., *J. Biol. Chem.* 272: 27618-27622 (1997); Raso, V. *Anal. Biochem.* 222:297-304 (1994); Raso et al., *Cancer Res* 41: 2073-2078 (1981); Raso et al., Monoclonal antibodies as cell targeted carriers of covalently and non-covalently attached toxins. *In* Receptor mediated targeting of drugs, vol. 82. G. Gregoriadis, G. Post, J. Senior and A. Trouet, editors. NATO Advanced Studies Inst., New York. 119-138 (1984)) will attach to appropriate receptors on the luminal side of the vessel. Once bound to the receptor, both components of the bispecific antibody ~~can~~ pass across the blood-brain barrier by the process of transcytosis. Anti-β-amyloid antibodies which have entered the brain interact directly with both β-amyloid plaques and the soluble β-amyloid pool. It has been estimated that concentrations of macromolecules in the 10^{-8}-10^{-7}M range can be achieved in the brain using vector-mediated delivery via these brain capillary enriched protein target sites (Maness et al., *Life Sciences* 55: 1643-1650 (1994); Lerner et al., *Science* 252: 659-667 (1991)). Importantly, the vector appears safe since animals dosed daily for two weeks with an anti-transferrin receptor antibody ~~showed~~ no loss of integrity of the blood-brain barrier using a radioactive sucrose probe (Broadwell et al., *Exp Neurol 142:* 47-65 (1996)).

The Exemplification details the production of vectorized bispecific antibodies which bind β-amyloid. The bispecific

antibodies transcytose across the blood brain barrier via a first specificity which binds the transferrin receptor. Use of antibodies which bind the transferrin receptor for delivery of agents across the blood brain barrier is described by Friden et al. in U.S. Patents No. 5,182,107; No. 5,154,924; No. 5,833,988; and No. 5,527,527; the contents of which are incorporated herein by reference.

Results from experiments presented in the Exemplification section which follows indicate that the produced bispecific antibodies retain their separate specificities and are delivered across the blood-brain barrier into the brain parenchyma and brain capillaries of a live animal when administered intravenously.

Alterna~~tive~~l methods for the production of bispecific antibodies have been described for genetically engineering bispecific reagents or for producing them intracellularly by fusing the two different hybridoma clones **[VR: PLEASE PROVIDE REFERENCES FOR THESE METHODS]**. Vectorized bispecific antibodies produced by these techniques can also be used in the methods of the present invention.

Since ~~Because~~ the introduction of whole antibodies into the brain might be detrimental if they were to fix complement and promote complement-mediated lysis of neuronal cells, it may be beneficial to produce and utilize smaller vectorized $F(ab')_2$ bispecific reagents. ~~can be produced~~. It has been shown that aggregated β-amyloid itself can fix complement in the absence of any antibody and that the resulting inflammation may contribute to the pathology of Alzheimer's disease. The possibility of intracerebral antibody having a similar effect can be ~~would be~~ greatly reduced by eliminating the Fc region of the antibody. Moreover, since coupling of Fab' halves uses the intrinsic hinge region cysteines, no extraneous substituent linkage groups need ~~to~~ be added. Faster or more efficient entry into the brain represents another potential advantage that smaller $F(ab')_2$ or Fv_2 reagents may provide for intracerebral

delivery. In addition, the two types of vectorized molecules may have different biodistribution and plasma half-life characteristics (Spiegelberg et al., *J. Exp. Med.* 121: 323 (1965)). 7 re-write

Depending on their design, anti-β-amyloid bispecific antibodies ~~situated~~ in the brain potentially ~~can~~ function by ~~in three different ways~~ mechanisms can to reduce soluble β-amyloid and β-amyloid deposits. by 3 potential mechanisms. 3 different ~~An~~ anti-β-amyloid bispecific antibody that tightly binds soluble β-amyloid will not only sequester the peptide but, due to efflux of vectorized molecules from the central nervous system (Kang et al., *J. Pharm. Exp. Ther.* 269: 344-350 (1994)), ~~potentially can~~ may also carry the bound ~~Aβ~~ β-amyloid out of the brain and release it into the blood stream. Such a ~~This~~ clearance mechanism would lead to a continuous cycling of β-amyloid out of the brain. add catalytic Abs 7

To be effective the anti-β-amyloid sites of a bispecific antibody must be empty before passage out of the blood and into the brain. Therefore the concentration of bispecific antibody in animals must exceed the level of β-amyloid circulating in the blood. Calculations performed based upon known β-amyliod levels (Scheuner et al., *Nature Med. 2*: 864-870 (1996)) and a medium-range plasma level of bispecific antibody expected in a treated animal indicated 99.9% of the bispecific antibodies that enter the brain will have unoccupied anti-~~Aβ~~ β-amyloid combining sites.

Another way of delivering antibodies to the brain is via direct infusion of anti-β-amyloid antibodies into the brain of an animal. This technique gives these antibodies immediate access to β-amyloid in the brain without having to cross the blood-brain barrier. Direct infusion can be accomplished via direct parenchymal or intracerebroventricular infusion (Knopf et al., *J Immunol. 161*: 692-701 (1998)). Briefly, the animal is anesthetized and placed in a stereotaxic frame. A midsaggital incision is made on the scalp to expose the skull and the underlying fascia is scraped away. A hole is drilled to accept a sterilized length of stainless steel hypodermic tubing, which is

stereotaxically advanced so that its tip is appropriately located in the brain. A guide cannula is then attached to the skull and sealed. The cannula remains in place for multiple infusions of antibody into the brain. A bolus of a sterile 50 mg/ml solution of a monoclonal anti-β-amyloid can be infused over a 2-8 minute period into an immobilized animal via an injection cannula.

Delivery of catalytic antibodies into the brain of an animal via one of the above described methods, can also be used to disaggregate amyloid plaques present in the brain. The advantage of delivering an β-amyloid-specific catalytic antibody into the brain is two-fold. The β-amyloid peptide is permanently destroyed by such antibodies and, since catalysis is continuous, each antibody inactivates many target β-amyloid molecules in the brain. Thus much less vectorized bispecific antibody has to be delivered into the central nervous system to achieve the desired depletion of β-amyloid.

The amount of antibody to be administered or delivered to the animal should be sufficient to cause a significant reduction in β-amyloid levels in the brain of the animal. The appropriate amount will depend upon various parameters (e.g. the particular antibody used, the size and metabolism of the animal, and the levels of endogenous β-amyloid) and is to be ~~should be~~ determined on a case by case basis. Such determination is within the means of one of average skill in the art through no more than ~~the course of~~ routine experimentation.

It is expected that additional benefits with respect to lowering brain β-amyloid levels and preventing or disaggregating amyloid plaques can be achieved through utilizing a combination of one or more of the above described approaches.

Exemplification

SECTION

~~PART~~ 1: RETENTION OF β-AMYLOID IN THE CIRCULATION

Synthesis of β-Amyloid Peptide Antigens

The amino acid sequence of the 43 residue β-amyloid peptide (Aβ)is listed in Figure 1. To determine which sites on this Aβ peptide were best suited for antibody-mediated therapy, three key regions (amino-terminal, central and carboxy-terminal) of the Aβ 43-mer were chosen to generate epitope-specific vaccines. These shortened peptides served as antigenic epitopes to induce a highly specific antibody response.

Monoclonal antibodies to the amino-terminal region of Aβ have been shown in the past to have the ability to solubilize Aβ aggregates (Solomon et al., *Proc. Natl. Acad. Sci. USA* 94(8): 4109 (1997))(Solomon et al., *Proc. Natl. Acad. Sci. USA* 94(8): 4109 (1997); Solomon et al., *Proc. Natl. Acad. Sci. USA* 93(1): 452 (1996)). for the present experiments, A peptide consisting of the amino-terminal region of Aβ was similarly designed for the present experiments (shown in Fig. 2 and listed in SEQ ID NO: 2) and used to elicit amino-terminal specific antibodies that bind Aβ. A Cys residue was added to the C-terminus of the Aβ sequence to provide a suitable linkage group for coupling this peptide to an antigenic carrier protein such as maleimide-activated Keyhole Limpet Hemocyanin (KLH).

A peptide encompassing the central region of Aβ was also synthesized (shown in Fig~~ure~~. 3 and listed in SEQ ID NO: 3). A Cys residue was placed at the N-terminus of the Aβ sequence to provide a sulfhydryl linkage group for coupling the peptide to antigenic maleimide-activated carrier proteins such as KLH.

To produce an antigen for eliciting an immune response directed against the carboxy-terminus of Aβ (Suzuki et al., *Science* 264:1336(1994)), a decapeptide encompassing the N-terminal region of Aβ, with an additional Cys residue at the N-terminus, was synthesized (Shown in Fig. 4, and listed in SEQ ID

NO: 4). The Cys substitution was designed to provide a sulfhydryl linkage group for coupling the peptide to antigenic a ~~maleimide-activated~~ carrier proteins such as KLH.

Coupling the peptides to an antigenic carrier protein

The different Cys containing Aβ peptides were individually thioether-linked to maleimide-activated KLH. A multivalent Aβ vaccine was also produced by simultaneously linking all three of these peptides to maleimide-activated KLH. In addition the full-length Aβ 43-mer was linked to KLH using glutaraldehyde.

Antibodies Elicited with the β-Amyloid Vaccines

Normal BALB/c mice were immunized by standard procedures with the KLH-linked Aβ vaccines described above. The mice were either bled or sacrificed for removal of the spleen for hybridoma production. Sera and monoclonal antibodies obtained were characterized for binding to Aβ.

Table 1 shows the results from an ELISA run with 1/100 diluted serum from two non-immunized control mice versus 1/100 and 1/1000 diluted serum from a mouse that was immunized with a central region Aβ peptide-KLH vaccine. The free Aβ peptide was adsorbed directly onto the microtitre plate to avoid detection of anti-KLH ~~antibodies in~~ the serum. Monoclonal antibodies raised have also been against this central region Aβ ~~peptide and have also been successfully identified~~ using ~~this~~ assay. ELISA and produced Abs by hybridoma fusion were identified okay

Table 1 ELISA for Binding to the Central Region Aβ Peptide

Addition		Antibody Bound (O.D. 450nm)
Control Serum A	1/100	0.666
Control Serum B	1/100	0.527
Mouse 1 antiserum	1/100	3.465

Mouse 1 antiserum	1/1000	2.764

A binding assay was performed to determine whether the monoclonal anti-$A\beta$ antibodies identified ~~by the above assays~~ also bound to the full length $A\beta$ peptides. $^{125}I\text{-}A\beta_{1\text{-}43}$ probe was incubated with hybridoma secretions from the indicated clones. A standard polyethylene glycol separation method was used to detect $^{125}I\text{-}A\beta_{1\text{-}43}$ bound antibody (Table 2). Results presented in Table 2 indicate that the antibodies generated to the peptide fragments also bound full length $A\beta_{1\text{-}43}$.

Table 2 $^{125}I\text{-}A\beta_{1\text{-}43}$ Binding Assay

Addition		$^{125}I\text{-}A\beta_{1\text{-}43}$ Bound (cpm)
Control Hy		3,171
Control Hy		2,903
6E2		15,938
6E2	1/10	9,379
3B1		12,078
3B1	1/10	3,353
8E3		10,789
8E3	1/10	3,249

It was recently reported that when $^{125}I\text{-}A\beta_{1\text{-}40}$ is added to human plasma, ~89% binds to albumin (Biere et al., *Journal of Biological Chemistry* 271(51):32916 (1996)). This raises the concern that the ~~This reported result suggests that the observed albumin~~ serum albumin will interfere w/ antibody binding. Binding assays were performed in the presence and absence of serum albumin, to determine whether albumin binding ~~would~~ interferes with antibody

binding to Aβ. The ability of purified 5A11 monoclonal anti-Aβ antibody to bind ^{125}I-$A\beta_{1-40}$ was unaffected by the presence of human serum albumin (HSA) at 60 mg/ml, even though this was a 500-fold molar excess over the antibody concentration (Table 3). These results indicate that the ability of antibodies to bind to and sequester Aβ in the blood will not be attenuated by the presence of other binding proteins.

Table 3. ^{125}I-$A\beta_{1-40}$ Binding to Antibody in the Presence of Human Serum Albumin*

Addition	^{125}I-$A\beta_{1-40}$ Bound (cpm)	Specifically Bound (% of total added)
Control	8,560	-
+ 5A11 anti-Aβ	64,589	79
Control + HSA*	3,102	-
+ 5A11 anti-Aβ + HSA*	55,304	75

*HSA at 60 mg/ml (~1 mM); anti-Aβ 5A11 at $2x10^{-6}$ M; Added ~70,000 cpm of ^{125}I-$A\beta_{1-40}$

analog at an amide linkage) discussed further in section II

Monoclonal Antibody Production

A mouse was immunized had a with a KLH conjugate of the central region (This peptide ~~also possessed a~~ phenylalanine statine analog transition state ~~mimic~~ of the central region $A\beta_{10-25}$ peptide. A hybridoma fusion was performed and the resulting monoclonal antibodies analyzed to characterize the specificity of the immune response to the vaccine. Hybridoma supernatants produced in the fusion were screened using ELISA to assess their binding to the $A\beta_{1-43}$ peptide.

The monoclonal antibodies produced were determined to bind to the $A\beta_{1-43}$ peptide adsorbed directly onto an ELISA plate. Strong color reactions were obtained in this ELISA using only 10 μl of hybridoma supernatant while the addition of media alone produced low background color. These results indicate that the

antibodies not only bound to the small peptide immunogen but they were also reactive with the full-length $A\beta_{1-43}$. Importantly, antibodies bound to the carrier-free $A\beta$ peptide adsorbed directly onto microtitre plates, showing their specificity for the peptide rather than the immunogenic carrier. The high affinity 5A11 monoclonal antibody (Table 3) was obtained from this hybridoma fusion. **[VR: THIS IS MISLEADING. PLEASE RECTIFY THIS STATEMENT WITH THE FACT THAT ANTIBODY 5A11 WAS OBTAINED FROM IMMUNIZATION WITH A TRANSITION STATE MIMIC PEPTIDE ANTIGEN.]**

A second mouse was immunized with a KLH conjugate of the $A\beta_{35-43}$ analog encompassing the C-terminal region of $A\beta$. Serum from the mouse was screened for reaction with $A\beta_{1-43}$ adsorbed directly onto the ELISA wells. The assay results are presented in Table 4. The spleen of this mouse was then used for a hybridoma fusion to further characterize the specificity of its immune response. Importantly, none of the mice immunized with $A\beta$ vaccines or the anti-$A\beta$ ascites-producing mice displayed ill effects even though some of those induced antibodies cross-react with mouse $A\beta$ and mouse amyloid precursor protein.

Table 4 ELISA for Binding of Antiserum Directed to the Carboxy-terminal $A\beta$ Peptide

	Antibody Bound (O.D. 450nm)
Addition	Native $A\beta_{1-43}$
Control Serum	0.484
Mouse Antiserum	1.765

Monoclonal antibodies from hybridoma clones generated above were screened for binding to the small carboxy-terminal peptide $A\beta_{35-43}$ and the full-length $A\beta_{1-43}$. Results are presented in Figure 5. The monoclonal antibodies bound to the carboxy-

terminal locus on each of these carrier-free Aβ peptides adsorbed directly to the microtitre plate, confirming their specificity for the peptide rather than the immunogenic carrier. The clones were also tested with Aβ_{1-40} to identify antibodies which do not react with this shortened, 40 amino acid residue version of Aβ and thus will specifically bind to the carboxy-terminus of Aβ_{1-43} (Fig.5). Used therapeutically, this vaccine should elicit antibodies which will preferentially bind the less abundant but more noxious Aβ_{1-43} species in the blood, as opposed to the smaller and less detrimental Aβ_{1-40}.

<u>Antibodies Affect the Distribution of ^{125}I-Aβ in Normal Mice</u>

Anti-Aβ antibodies in the circulation cannot cross the blood-brain barrier to a significant extent and therefore should act as a sink that prevents ^{125}I-Aβ_{1-40} from reaching the brain. This retention effect was demonstrated by measuring the blood levels in mice 4 h after injecting them with equal amounts of ^{125}I-Aβ_{1-40} either alone or along with our 5A11 anti-Aβ monoclonal antibody (Table 5). The passage of ^{125}I-Aβ_{1-40} out of the peripheral circulation was greatly curtailed in animals which concomitantly received the specific anti-Aβ antibody. That finding extends the *in vitro* results with the 5A11 antibody (Table 3) by demonstrating that it can also effectively bind Aβ in an experimental animal. The ~~fact~~ observation that animals treat~~ment~~ed with this antibody ~~retained~~ 10-times more ^{125}I-Aβ_{1-40} in the circulation indicates that the equilibrium distribution of Aβ in the body can be dramatically altered by ~~125~~ selective sequestration in the blood.

Table 5 Anti-Aβ Antibody Impedes the Passage of ^{125}I-Aβ_{1-40} Out of the Circulation

^{125}I-Aβ_{1-40} in Blood

Mouse Injected With	(cpm/gm)
^{125}I-Aβ_{1-40} alone	27,300
^{125}I-Aβ_{1-40} + 5A11 anti-Aβ	278,900

Materials and Methods

Peptide synthesis. The 40mer Aβ_{1-40}, the 43mer Aβ_{1-43}, and the three small Aβ peptides Aβ_{1-16}, Aβ_{10-25}, and Aβ_{35-43}, were synthesized by standard automated Fmoc chemistry. Newly synthesized peptides were purified by HPLC and their composition was verified by mass spectral and amino acid analysis. The Aβ 43mer was obtained from a commercial source (Bachem, Torrance, CA).

Conjugation of β-amyloid peptides to immunogenic carriers. The small Aβ peptides were linked to the KLH carrier protein in order to render them antigenic. A Cys residue was strategically placed at the N- or C-terminal end of these Aβ peptides to provide a suitable linkage group for coupling them via a thioether bond to maleimide activated carrier proteins. This linkage is stable and attaches the peptide in a defined orientation. Addition of ~20 peptides/KLH is typically obtained by this conjugation method. The longer, full length Aβ peptides were linked to carrier proteins using a glutaraldehyde coupling procedure. ~~The outlined methods are an effective and expedient way of producing experimental vaccines for use in animals.~~

Immunization of Mice. Normal BALB/c mice were immunized by standard procedures with the KLH-linked Aβ vaccines described above. Briefly, mice were injected i.p. with antigen emulsified in complete Freunds adjuvant, followed by a second course in incomplete Freunds adjuvant. The mice were i.v. boosted with antigen in PBS three days prior to bleeding them or removing the spleen for hybridoma fusions to produce monoclonal antibodies.

None of the mice immunized with Aβ vaccines or the anti-Aβ ascites-producing mice displayed ill effects even though some of th~~ese~~e ~~induced~~ antibodies cross-reacted with mouse Aβ and mouse amyloid precursor protein.

ELISA. The presence of bound anti-peptide antibodies was revealed by using a peroxidase-labeled anti-mouse IgG probe followed by the chromogenic substrate (Engvall et al., *Immunochemistry* 8: 871-875 (1971).

Binding Assay. Both $A\beta_{1-43}$ and $A\beta_{1-40}$ were radiolabeled with ^{125}I. The iodinated peptide was separated from unlabeled material by HPLC to give essentially quantitative specific activity (~2000 Ci/mmol) (Maggio et al., *Proc. Natl. Acad. Sci.* 89:5462 (1992)). $^{125}I\text{-}A\beta_{1-43}$ probe was incubated for 1h at 23°C with Hy media taken from hybridoma clones producing monoclonal anti-Aβ antibodies. A standard polyethylene glycol separation method was used to detect the amount of $^{125}I\text{-}A\beta_{1-43}$ bound to antibody.

Section ~~PART~~ II: ELICITING MONOCLONAL ANTIBODIES WITH TRANSITION STATE ANTIGENS

Transition state peptide antigens

Different types of transition state peptide antigens were synthesized to use in the generation of antibodies which preferentially recognize (hydrolysis) transition states of Aβ at a predetermined amide linkage position.

A series of statine (Sta) transition state analogs encompassing the carboxy-terminal region of Aβ (Cys-Met-Val-Gly-Gly-Val/Sta-Val/Sta-Ile/Sta-Ala-Thr) were synthesized. Replacement of the proposed scissile peptide linkage between Val_{39} and Val_{40} ~~(4)~~, Val_{40} and Ile_{41} ~~(4)~~ and Ile_{41} and Ala_{42}, ~~(4)~~ with a "statyl" moiety ($-CHOH-CH_2-CO-NH-$) was designed to elicit

catalytic antibodies that hydrolytically cleave Aβ at one of these sites (Figure 6). A Cys residue was placed at the N-terminal position of these peptides to provide a suitable linkage group for coupling to a maleimide-activated carrier protein.

A series of phenylalanine statine (PhSta) transition state analogs encompassing the central region of Aβ (Cys-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe/PhSta-Phe/PhSta-Ala-Glu-Asp-Val-Gly-amide) was synthesized in this laboratory (Fig. 7).

Replacement of the proposed scissile peptide linkage between Phe_{19} and Phe_{20}) ~~(?)~~ and between Phe_{20} and Ala_{21} ~~(?)~~ with a statyl moiety (-CHOH-CH_2-CO-NH-) was designed to elicit catalytic antibodies that hydrolytically cleave Aβ at these sites (Figure 7). A Cys residue was placed at the C-terminus of these peptides to provide a sulfhydryl linkage group for coupling the peptides to antigenic, maleimide-activated carrier proteins ~~such as KLH~~.

A structural comparison (Fig. 8) was made between the native Aβ peptide and the transition state phenylalanine statine Aβ peptide using a graphics workstation. An energy minimization algorithm (2000 iterations) was applied to arrange each peptide in its most favorable conformation.

The peptide link -CO-NH- between Phe_{19} and Phe_{20} was replaced with an elongated "statyl" moiety -CHOH-CH_2-CO-NH- and an energy minimization was applied. This orientation shows the difference between the planar peptide link -CO-NH- of natural Aβ (left) versus the extended, tetrahedral "statyl" moiety -CHOH-CH_2-CO-NH- in the transition state peptide (right).

An antibody combining site complementary to a tetrahedral statine transition state analog will force the planar peptide bond of the Aβ substrate into a transition state-like conformation. Such distortion should catalyze the cleavage of Aβ at that locus in the peptide sequence.

A reduced peptide bond linkage can be easily placed at almost any site in the Aβ molecule to produce a reduced peptide bond transition state analog. This analog can also be used to

The possibility of using a reduced peptide bond linkage to mimic the ~~hydrolysis~~ transition state of hydrolysis of ~~that~~ an amide ~~peptide~~ bond ... was also

elicit catalytic antibodies that will hydrolytically cleave Aβ at the chosen site. The ~~first~~ reduced peptide bond transition state Aβ analog made was the (Gln-Lys-Leu-Val-Phe-CH_2-NH_2^+-Phe-Ala-Glu-Asp-Val-Gly-Cys-amide) central region peptide; [calculated 1,342 (M+1); observed 1,344].

A structural comparison (Fig. 9) was made between the native Aβ peptide and the reduced peptide bond transition state Aβ analog using a graphics workstation. An energy minimization algorithm (2000 iterations) was applied to arrange each peptide in its most favorable conformation. The peptide link -CO-NH- ~~between Phe~~$_{19}$ and Phe$_{20}$ was replaced with a reduced peptide bond -CH_2-NH_2^+- and an energy minimization was applied. The orientation shown indicates the difference between the planar peptide link -CO-NH- of natural Aβ (left) versus the corresponding tetrahedral moiety -CH_2-NH_2^+- in the reduced peptide bond transition state analog (right).

A phosphonamidate transition state analog of the carboxy-terminal region of Aβ has also been synthesized (Fig. 10). Replacement of the proposed scissile peptide linkage between Gly$_{38}$ and Val$_{39}$ (4) with a phosphonamidate moiety (-PO_2^--NH-) was designed to elicit catalytic antibodies that will hydrolytically cleave Aβ at this site. The N-acetyl-Cys residue was placed at the position of Leu$_{34}$ to provide a suitable linkage group for coupling this peptide to an antigenic carrier protein. The structures in Fig. 11 represent the putative transition state for peptide hydrolysis by zinc peptidases, ~~and~~ versus structure of the phosphonate and phosphonamidate mimics. Similar tetrahedral transition state intermediates are known to be formed by reaction with each of the four classes of proteolytic enzymes, the serine-, cysteine-, aspartic- and metallo-peptidases.

The synthesis of phosphonate Aβ transition state analog peptide (eg. N-acetyl-Cys-Met-Val-Gly-Gly-PO_2^--O-Val-Val-Ile-Ala-amide) will follow a similar scheme and will use some of the same intermediates described for the phosphonamidate transition state

analog.

A structural comparison was made between the native $A\beta$ peptide and the transition state phosphonamidate $A\beta$ peptide (Fig. 12) using a graphics workstation. The peptide link -CO-NH- between Gly_{38} and Val_{39} was replaced with a phosphonamidate bond -PO_2^--NH- and an energy minimization was applied. The orientation shown in Fig. 12 illustrates the difference between the planar peptide link -CO-NH- of native $A\beta$ (left) versus the corresponding tetrahedral phosphonamidate bond -PO_2^--NH- in the transition state peptide (right).

An antibody combining site complementary to the tetrahedral transition state analog on the right of Fig. 12, will force the normally planar bond of the $A\beta$ substrate peptide on the left into a transition state-like conformation. Such bond distortion was expected to catalyze the hydrolytic cleavage of the $A\beta$ peptide at the Gly_{38}-Val_{39} linkage.

<u>Immunization with transition state peptide antigens</u>

Peptide antigens were coupled to the immunogenic carrier KLH prior to immunization of mice.

Standard protocols were used to immunize the Tg2576 transgenic mice and BALB/c mice with the KLH-linked $A\beta$ peptides described in the preceding sections. Briefly this procedure used i.p. injection of the different antigens emulsified in complete Freunds adjuvant, followed by a second course in incomplete Freunds adjuvant. Three days prior to hybridoma fusion, the BALB/c mice were boosted i.v. with antigen in PBS.

A hybridoma fusion was performed using the spleen of a mouse immunized with the phenylalanine statine transition state $A\beta$-KLH antigen (Fig. 7) and also the statine (Sta) transition state analogs encompassing the carboxy-terminal region of $A\beta$ (Fig. 6) (Cys-Met-Val-Gly-Gly-Val/Sta-Val/Sta-Ile/Sta-Ala-Thr) **[SH:THESE WERE NOT THE ONLY TRANSITION STATE ANTIGENS USED TO GENERATE HYBRIDOMAS/MONOCLONAL ANTIBODIES. VR IS MAKING A TABLE CATALOGING**

THE DIFFERENT TS ANALOGS, SITES MODIFIED AND MABS GENERATED]

Demonstration of Aβ binding by generated antibodies

It was very important to demonstrate that the anti-Aβ and anti-transition state Aβ monoclonal antibodies bound to the natural $A\beta_{1-43}$ peptide which they were designed to sequester or cleave. To do this, $A\beta_{1-40}$ and $A\beta_{1-43}$ were radiolabeled with ^{125}I and the iodinated peptide were ~~then~~ separated from unlabeled material by HPLC. Probe was incubated with either purified anti-Aβ antibodies or media taken from hybridoma clones producing anti-Aβ antibodies. The amount of ^{125}I-$A\beta_{1-43}$ bound to antibody was determined using a polyethylene glycol separation method. Results of the experiment are presented in Table 6.

The data in Table 6 demonstrate the ability of the purified 5A11 monoclonal anti-Aβ antibody to bind a high percent of ^{125}I-$A\beta_{1-40}$. This binding assay was used to screen clones and purified antibodies (Table 6) for their ability to bind Aβ (~~below~~). Similar procedures can also serve as the basis for a competitive displacement assay to measure the relative binding strength of different unlabeled Aβ peptides (note: With very efficient catalytic antibodies this binding assay may have to be performed on ice to ensure that no cleavage of Aβ occurs during the 1h incubation time.) The assay ~~will~~ al~~low~~s the rapidly identified ~~ication of~~ clones ~~which~~ producing high affinity anti-Aβ antibodies.

Table 6 **^{125}I-$A\beta_{1-40}$ Binding to a Purified Monoclonal Anti-Aβ Antibody ***

Addition	^{125}I-$A\beta_{1-40}$ Bound (cpm)	Specifically Bound (% of total added)
Control	8,560	-
+ 5A11 anti-Aβ	64,589	79

* anti-Aβ 5A11 at $2x10^{-6}$ M; Added ~70,000 cpm of ^{125}I-Aβ_{1-40}

Monoclonal antibodies from ~~some of~~ the hybridomas ~~supernatants~~ obtained using the phenylalanine statine transition state Aβ-KLH antigen were screened ~~using~~ by ELISA to assess their binding to both the normal Aβ_{1-43} peptide and to the phenylalanine statine transition state Aβ peptide. Two major patterns were found (Fig. 13).

One group of antibodies (the left portion of Fig. 13) bound to the immunizing transition state peptide and cross-reacted strongly with the native Aβ_{1-43} peptide (~~when~~ each was adsorbed directly onto the ELISA plate). ~~A~~ The second group (the right portion) showed a high binding preference for the phenylalanine statine transition state Aβ peptide and reacted minimally with native Aβ_{1-43}.

Strong color reactions were obtained in this ELISA using only 10 μl of hybridoma supernatant while Hy media alone or PBS gave a low background (Fig. 13). These results demonstrate that the comparative ELISA screen, although only a semi-quantitative measure of binding, provides a means for ~~choosing~~ identifying monoclonal antibodies that are highly selective for, and most reactive with, the transition state. Importantly, ~~the antibodies bound to the~~ experiment was performed with carrier-free Aβ peptides adsorbed directly onto microtitre plates, ~~showing~~ indicating ~~their~~ antibody specificity for ~~the~~ peptide, rather than the carrier

These findings indicate that several of the generated anti-Aβ transition state antibodies were unique. They bound to both the phenylalanine statine- and normal-Aβ peptides. Their selective recognition of the transition state and weaker cross-reaction with native Aβ_{1-43} however, indicates that this binding interaction is very different from that shown by conventional anti-native Aβ antibodies. It further indicates that these new antibodies may be able to force the native Aβ peptide into a conformation resembling the transition state for hydrolytic cleavage. Importantly, some of the antibodies which showed only

minimal binding to $A\beta_{1-43}$ in this ELISA, did display cross-reactivity with the natural peptide using a highly sensitive ^{125}I-$A\beta_{1-43}$ binding assay (Table 6).

ELISAs were also performed to investigate the binding of anti-statine analog antibodies to both the normal $A\beta_{1-43}$ peptide and to the statine transition state $A\beta$ peptide (Fig. 14). The antibodies bound to the C-terminal locus on these carrier-free $A\beta$ peptides (adsorbed directly to the microtitre plate) confirming their anti-peptide specificity. Most of the antibodies preferentially recognized the statine $A\beta$ transition state but cross-reacted with native $A\beta_{1-43}$. This indicates that these new antibodies are able to force the native $A\beta$ peptide into a conformation resembling the transition state for hydrolytic cleavage of its C-terminal amino acids. Such cleavage is predicted to convert $A\beta_{1-43}$ into potentially less harmful shorter peptides, like $A\beta_{1-40}$ or $A\beta_{1-39}$.

Clone 11E9 had the strongest preference for the statine analog and may be the most likely to have catalytic activity (Fig. 14). Several clones displayed no difference in their reactivity with the native versus statine transition state $A\beta$ peptide. The clones were also tested with $A\beta_{1-40}$ to identify antibodies which do not react with this shortened, 40 amino acid version of $A\beta$ (Fig. 14). Used therapeutically, such antibodies should preferentially bind/cleave the less abundant but more noxious $A\beta_{1-43}$ species in the blood, as opposed to the smaller and less detrimental $A\beta_{1-40}$.

Solid phase and TLC $A\beta$ proteolytic assays

A solid phase ^{125}I-labeled $A\beta$ assay was developed to screen anti-transition state antibody hybridoma supernatants for specific proteolytic activity. The peptide Cys-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Tyr-amide (SEQ ID NO: 5) which encompasses amino acids 14-25 of $A\beta$ was radiolabeled and coupled

to a thiol-reactive, iodoacetyl-Sepharose gel to form an irreversible linkage. The product was contacted with anti-transition state antibody and assayed for the progressive release of soluble ^{125}I-peptide from the solid phase matrix. Release of radioactivity from the ^{125}I-Aβ-Sepharose was used to identify catalytic activity (Fig. 15). The assay was verified by the ability of several different proteases to rapidly hydrolyze this Sepharose-linked Aβ substrate. The peptide was readily accessible to proteolytic cleavage as revealed by a release of soluble ^{125}I-peptide that increased with incubation time.

The results presented in Figure 15 indicate that the antibody-containing media of several clones released ^{125}I-peptide at a greater rate than other clones from this fusion or the PBS and Hy medium controls. Large amounts of these antibodies can be obtained, purified and tested at higher concentrations to achieve much faster rates of cleavage and to verify that the antibodies are acting in a catalytic mode using conventional enzyme kinetics. By changing the composition of the ^{125}I-peptide this same strategy can be used to assay antibodies reactive with different regions of Aβ.

A thin layer chromatography-based autoradiography assay was devised to obtain more definitive evidence for antibody-mediated cleavage of Aβ. Selected anti-phenylalanine statine Aβ transition state clones were expanded and ascites production induced. The different monoclonal antibodies were isolated using protein A-Sepharose. Two ^{125}I-labeled peptides, ^{125}I-Aβ_{1-40} and a 17-mer, encompassing amino acids 9-25 of Aβ, were used to test for peptide cleavage. The antibodies were added to the ^{125}I-peptides, allowed to incubate and the reaction mix spotted onto polyamide thin layer sheets which were then developed in different solvents. The migration of ^{125}I-products was followed by exposing the sheet using a quantitative phosphoimager system. Quantitation of the different ~~sized~~ labeled peptide fragments produced indicated that addition of the antibodies to the Aβ

peptides lead to significant break down of the Aβ peptides compared to the untreated peptides (PBS).

Disaggregation of β-amyloid by monoclonal antibodies

has been shown previously to

The self-aggregation of synthetic Aβ peptides ~~leads~~ lead to microscopic structures resembling amyloid plaques in the brain (Solomon et al., *Proc. Natl. Acad. Sci. USA* 94: 4109-12 (1997); Solomon et al., *Proc. Natl. Acad. Sci. USA* 93: 452-5 (1996)) which exhibit the same bright green fluorescence upon exposure to thioflavin T. These aggregates are very stable and usually require harsh detergents or strong acids to dissolve. However, it has been demonstrated that the binding of certain anti-Aβ monoclonal antibodies can effectively inhibit the initial aggregation of this peptide and also disaggregate preformed Aβ complexes (Solomon et al., *Proc. Natl. Acad. Sci. USA* 94: 4109-12 (1997); Solomon et al., *Proc. Natl. Acad. Sci. USA* 93: 452-5 (1996)).

A radioactive assay was used to quickly screen the different monoclonal antibodies ~~produced for~~ those produced by the present experiments for an ability to dissolve preformed Aβ aggregates, made with ^{125}I-labeled and unlabeled soluble Aβ peptide. An aliquot of the labeled aggregate was incubated with either PBS, the 5A11 anti-Aβ antibody, or an equal amount of an irrelevant mouse antibody (7D3, anti-human transferrin receptor), and the level of released radioactivity was subsequently measured (Table 7). ~~The fact that~~ The Aβ-specific 5A11 antibody solubilized 80% of the Aβ aggregates while an equal amount of the control antibody had only a minor effect, suggests that the equilibrium was displaced by antibody-mediated binding of soluble Aβ.

Table 7 Solubilization of ^{125}I-Aβ_{1-40} Aggregate by Monoclonal Anti-Aβ Antibody

Addition	^{125}I-Aβ_{1-40} in Ppt. (cpm)	Amount Solubilized (% of PBS Control)

PBS control	3,420	-
+ 5A11 anti-Aβ	676	80
+ 7D3 anti-TfR	2,458	27

Production of Vectorized Anti-Aβ/Anti-Receptor Bispecific Antibodies

Anti-Aβ antibodies were linked to anti-transferrin receptor antibodies (anti-TfR) which served as vectors for delivery of the anti-Aβ antibodies into the brain. The 7D3 mouse monoclonal antibody was used as the anti-TfR part of the construct. 7D3 is specific for the human receptor and selectively immunostains cortical capillaries in normal human brain tissue (Recht et al., *J Neurosurg* 72: 941-945 (1990)). Antibody attachment to the receptor is not blocked by an excess of human transferrin. The epitope recognized by this antibody is therefore distant from the receptor-ligand binding site. Bispecific antibodies constructed with this 7D3 antibody and an anti-Aβ antibody are predicted to be useful for therapy in patients with Alzheimer's disease. ~~Stained with the 7D3 Anti-TfR (Recht et al., *J Neurosurg* 72:941-945 (1990)) and possibly for preclinical trials in primates.~~

For studies in the transgenic mouse model of Alzheimer's disease an anti-mouse transferrin receptor monoclonal antibody produced in the rat was obtained. This antibody also appears to recognize a transferrin receptor epitope which does not involve ligand binding. The antibody therefore has no effect on cell proliferation when ~~tested~~ using murine lines.

A series of functional assays were performed after completion of the synthesis, purification and size analysis of the anti-Aβ/anti-transferrin receptor bispecific antibodies. The vectorized bispecific antibody, composed of a rat monoclonal antibody directed against the mouse transferrin receptor plus the 5A11 mouse anti-Aβ monoclonal antibody, was tested for the ability to attach to transferrin receptor bearing human cells.

Both components of the bispecific antibody were detected on the cell membrane by cytofluorimetry (Fig. 16) when this duplex was reacted with transferrin receptor positive mouse cells and probed using either a rat IgG-specific or mouse IgG-specific fluorescent secondary antibody reagent.

The capacity of the hybrid reagent to bind ^{125}I-Aβ compared favorably with that of the parent anti-Aβ antibody (Table 8).

Table 8 **^{125}I-Aβ Binding to Bispecific Antibody**

Addition	^{125}I-Aβ_{1-40} Bound (cpm)
Control	4,199
+ anti-Aβ	23,301
+ anti-Aβ/anti-receptor	22,850

To ensure that both of these binding activities resided on the bispecific antibody, transferrin receptor positive cells were treated with the hybrid reagent, unbound material was washed away, and then the cells with bound antibody was exposed to ^{125}I-Aβ_{1-40}. After washing away unbound Aβ, the cell-bound radioactivity was compared to control cells which had been identically prepared except for omission of pretreatment with bispecific antibody. The results are presented in Table 9, and verify the dual specificity of this bispecific antibody by clearly showing that it can simultaneously attach to the cell membrane and bind ^{125}I-Aβ_{1-40}.

Table 9 Bispecific Antibody-Mediated Binding of ^{125}I-Aβ to Receptor-Positive Cells

Pretreatment of Cells	^{125}I-Aβ_{1-40} Bound (cpm)
None	2,367
+ anti-Aβ/anti-transferrin receptor	11,476

Transcytosis of bispecific antibody into the brain

A rat monoclonal anti-mouse transferrin receptor antibody was coupled to a mouse monoclonal antibody (obtained from American Type Culture Collection (ATCC TIB 219), also designated R17 217.1.3 (Cell. Immunol. 83: 14-25 (1984))) so that the entry of this new vectorized bispecific construct into brain could be monitored. The bispecific antibody was labeled with ^{125}I and injected i.v. into normal mice. After different lengths of time the mice were sacrificed and the amount of ^{125}I-bispecific antibody that crossed the blood-brain barrier and entered the brain was gauged by a mouse capillary depletion method (Friden et al., *J. Pharm. Exper. Ther.* 278:1491-1498 (1996); Triguero et al., *J. Neurochem.* 54: 1882-1888 (1990)).

The amount of vectorized bispecific antibody found in the brain parenchyma or brain capillary fractions was measured following differential density centrifugation of the brain homogenate. These values were plotted as a function of time after i.v. injection (Fig. 17). The time-dependent redistribution of radiolabeled bispecific antibody from the capillaries and into the parenchyma was consistent with its passage across the cerebral endothelial blood-brain barrier (Joachim et al., *Nature* 341: 6239:226-30 (1989)). Even greater accumulation in the parenchyma is expected to occur if the antibodies attach to Aβ in the cerebral plaques of plaque-bearing mice.

<u>Monitoring the brain distribution of bispecific antibody in live mice</u>

The ability to follow the entry and accumulation of vectorized bispecific antibodies in the brain of live mice would greatly assist in the development of the intracerebral treatment of plaque-bearing mice. Such a development would enable time-course studies and would greatly reduce problems with inter-mouse variability. Preliminary studies with ^{125}I-labeled bispecific antibodies were performed to determine if immunoscintigraphy was feasible in this system. As a first step, either the radiolabeled vectorized bispecific antibody (^{125}I-R17/5A11) or a non-vectorized control bispecific antibody were administered i.v. to separate mice. Sequential brain images were accumulated at 1, 6, 24 and 48 hours following i.v. administration of the ^{125}I-labeled bispecific antibody probes. Although this technique suffered from a difficulty in determining how much of the signal was due to the levels of blood-borne radioactivity circulating through the brain, significant distinctions were noted in the brain of mice treated with the mouse transferrin receptor reactive bispecific antibody versus those receiving the control bispecific antibody. When the vectorized agent was used, brain levels increased between 1 and 6 hrs and then declined to a much lower level at 24 and 48 hrs. Mice treated with the control displayed no increase between 1 and 6 hrs. The reason for decreased brain levels at 24 hrs and beyond is not known but might be due to dehalogenation of the bispecific antibody probes so that free ^{125}I is released ~~which exits the brain.~~ Alternative methods utilizing radioactive labels such as ^{111}In (Sheldon et al., *Nucl. Med. Biol.* 18:519-526 (1991)) or ^{99m}Tc (Texic et al., *Nucl. Med. Biol.* 22:451-457 (1995)) attached to the vectorized bispecific antibody can be utilized in future experiments if the use of iodine presents a technical problem. This imaging technology will be useful for determining if smaller vectorized bispecific antibodies (eg. $F(ab')_2$) with different physical properties and an altered

biodistribution will penetrate into the brain more effectively.

$F(ab')_2$ heterodimers for vector-mediated transport into the brain

The introduction of whole antibodies into the brain might be detrimental if they were to fix complement and promote complement-mediated lysis of neuronal cells. The development of smaller vectorized $F(ab')_2$ bispecific reagents is expected to avoid this problem. It has been shown that aggregated Aβ itself can fix complement in the absence of any antibody and that the resulting inflammation may contribute to the pathology of Alzheimer's disease. The possibility of intracerebral antibody having a similar effect would be greatly reduced by eliminating the Fc region of the antibody. Moreover, since coupling of Fab' halves uses the intrinsic hinge region cysteines, no extraneous substituent linkage groups need to be added.

Faster or more efficient entry into the brain represents another potential advantage that smaller $F(ab')_2$ or Fv_2 reagents provide for intracerebral delivery. Such modified bispecific agents can be prepared and compared to full-sized hybrid antibodies for their relative effectiveness in reaching the brain, crossing the blood-brain barrier, and affecting Aβ plaque development, by the methods described herein. It is important to note however that only minor differences were found when the capacity of differently-sized anti-transferrin receptor bispecific reagents for delivering toxins into cells by receptor-mediated endocytosis was compared (Raso et al., *J. Biol. Chem.* 272: 27623-27628 (1997)). This observation might indicate that little variation will be seen for transcytosis across the brain capillary endothelial cells which form the blood-brain barrier. At the very least however one would expect the two types of vectorized molecules to have different biodistribution and plasma half-life characteristics (Spiegelberg et al., *J. Exp. Med.* 121: 323 (1965)).

Materials and Methods

Antigen synthesis. The statine and phenylalanine statine transition state peptides were synthesized using automated Fmoc chemistry. Fmoc-statine (Sta), [N-Fmoc-(3S,4S)-4-amino-3-hydroxy-6-methyl heptanoic acid] and Fmoc-"phenylalanine statine" (PhSta), [N-Fmoc-(3S,4S)-4-amino-3-hydroxy-5-phenylpentanoic acid] were purchased commercially. Each peptide was tested for purity by HPLC and its composition was verified by mass spectral and amino acid analysis. The design strategy and methods for synthesizing phosphonamidate- and phosphonate-based transition state peptides are straightforward (Bartlett et al., *Am. Chem. Society* 22:4618-4624 (1983); Bartlett et al., *Biochemistry* 26:8553-8561 (1987)). The N-terminal portion of the peptide (N-acetyl-Cys-Met-Val-Gly) was made using standard automated Fmoc chemistry. After cleavage from the resin the N-acetyl tetrapeptide was treated with pyridine disulfide to protect its sulfhydryl group. An acid chloride of Cbz-glycine phosphonate monomethyl ester (Bartlett et al., *Am. Chem. Society* 22:4618-4624 (1983); Bartlett et al., *Biochemistry* 26:8553-8561 (1987)) was coupled with Val-Val-Ile-Ala-amide which was synthesized by automated Fmoc chemistry. The last amino acid of Aβ, Thr, was omitted due to potential problems with its unprotected hydroxyl group. The product, Cbz-Gly-PO_2^--NH-Val-Val-Ile-Ala-amide has a phosphonamidate (methyl ester) bond between the Gly and Val residues. Next, the Cbz blocking group was removed using hydrogen so that the protected N-acetyl-Cys-Met-Val-Gly peptide could be added to the amino terminal end of this transition state peptide by HBTU-activated peptide linkage. Treatment with mercaptoethanol and rabbit liver esterase was used to deblock the peptide. Each key component in the synthetic scheme was tested for purity by HPLC and its composition was verified by mass spectral and amino acid analysis.

A reduced peptide bond linkage was placed at the indicated sites in the Aβ molecule. Automated Fmoc chemistry was used to begin synthesis of the peptide. A

pre-synthesized Fmoc amino aldehyde was then added manually and after the imide was reduced, automated synthesis was resumed (Meyer et al., *J. Med. Chem.* 38:3462-3468 (1995)).

Coupling of antigen to carrier. The native and transition state Aβ peptides were coupled to maleimide-activated KLH by standard procedures (Partis et al., J. Pro. Chem 2: 263-277 (1983), in order to elicit an immune response. A Cys residue was strategically placed at the N- or C-terminal end of the peptides to provide a suitable linkage group for coupling them via a thioether bond to maleimide activated carrier proteins. This stable linkage attaches the peptide in a defined orientation. Addition of ~20 peptides/KLH has been obtained based upon the transition state amino acid content as determined by amino acid analysis of the hydrolyzed conjugates (Tsao et al., *Anal. Biochem. 197*: 137-142 (1991)).

Immunization of mice. Standard protocols were used to immunize the Tg2576 transgenic mice and BALB/c mice with the KLH-linked Aβ peptides described in the preceding sections. Briefly this procedure used i.p. injection of the different antigens emulsified in complete Freunds adjuvant, followed by a second course in incomplete Freunds adjuvant. Three days prior to the hybridoma fusion, the BALB/c mice were boosted i.v. with antigen in PBS.

Aβ antigens will be emulsified in complete Freunds adjuvant and injected i.p. into BALB/c mice. After ~1 month animals were given a boost i.p. using the antigen emulsified with incomplete adjuvant. Serum from these animals was analyzed for anti-peptide antibodies by ELISA. BALB/c mice showing abundant antibody production were boosted by an i.v. injection with antigen and three days later they were used to generate hybridoma clones that secrete monoclonal antibodies.

None of the mice immunized with Aβ vaccines or the anti-Aβ ascites-producing mice displayed ill effects even though some of

those induced antibodies cross-react with mouse Aβ and mouse amyloid precursor protein.

Hybridoma production I. A hybridoma fusion was performed using the spleen of a mouse immunized with the phenylalanine statine transition state Aβ-KLH antigen. Spleen cells from mice with the highest titre were fused with mouse myeloma NS-1 cells to establish hybridomas according to standard procedures (Köhler et al., *Nature* 256:495 (1975); R. H. Kennett, Fusion Protocols. Monoclonal Antibodies, eds. R.H. Kennett, T.J. McKearn and K.B. Bechtol. Plenum Press, New York. 365-367 pp. (1980)).

^{125}I-Aβ binding assay. $A\beta_{1-40}$ and $A\beta_{1-43}$ were radiolabeled with ^{125}I and the iodinated peptide then separated from unlabeled material by HPLC to give quantitative specific activity (~2000 Ci/mmol) (Maggio et al., *Proc. Natl. Acad. Sci.* 89:5462-5466 (1992)). This probe was incubated for 1h at 23^{0}C with either purified anti-Aβ antibodies or media taken from hybridoma clones producing anti-Aβ antibodies. A polyethylene glycol separation method was used to detect the amount of ^{125}I-$A\beta_{1-43}$ bound to antibody. By using serial dilution, this assay can provide relative binding affinities for the different hybridoma supernatants or purified antibodies.

Solid phase Aβ proteolytic assay. A solid phase ^{125}I-labeled Aβ assay was developed to screen anti-transition state antibody hybridoma supernatants for specific proteolytic activity. The Cys-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Tyr-amide peptide (SEQ ID NO:5) encompassing amino acids 14-25 of Aβ was radiolabeled with ^{125}I and the iodinated peptide was then separated from unlabeled material by HPLC. The highly radioactive Aβ peptide was coupled to a thiol-reactive, iodoacetyl-Sepharose gel to form an irreversible linkage. Antibodies were added to the labeled Aβ, which was then assayed

for progressive release of soluble ^{125}I-peptide from the solid phase matrix at pH 7, 25°C. This assay was verified by the ability of several different proteases in to rapidly hydrolyze this Sepharose-linked Aβ substrate. Release of soluble ^{125}I-peptide increased with incubation time.

Although Aβ is cleaved by several naturally occurring proteases, preliminary tests indicated that interference from high levels of background hydrolysis was not a problem when assaying hybridoma supernatants of clones that did produce catalytic antibodies. A further precaution that can be taken against exogenous proteases is carrying out all hybridoma cell fusions and cell culturing in serum-free media.

TLC Aβ proteolytic assay. A thin layer chromatography-based autoradiography assay was used to obtain more definitive evidence for antibody-mediated cleavage of Aβ. Selected anti-phenylalanine statine Aβ transition state clones were expanded and ascites production induced. The different monoclonal antibodies were isolated using protein A-Sepharose. The cleavage assay used ^{125}I-Aβ_{1-40} and an ^{125}I-labeled 17-mer, encompassing amino acids 9-25 of Aβ. Binding of the two ^{125}I-labeled peptides to the purified monoclonal antibodies 5A11 and 6E2 was examined using either a PEG precipitation assay or by a co-electrophoresis method. Peptide cleavage was tested by adding the antibodies to the ^{125}I-peptides, incubating and then spotting the reaction mix onto polyamide thin layer sheets. The chromatographs were developed in different solvents (eg. 0.5 N HCl, 0.5 N NaOH or pH 7 phosphate buffer) and the migration of ^{125}I-products was followed by exposing the sheet using a quantitative phosphoimager system.

Screen and isolate select anti-Aβ antibodies. An ELISA was used to initially screen for anti-Aβ and anti-transition state Aβ peptide monoclonal antibodies. Both the transition state peptide

and the corresponding natural Aβ peptide were adsorbed onto separate microtitre plates. The hybridoma supernatants were screened using two assays so that the relative binding to both native and transition state Aβ peptides could be quantitated. Clones producing monoclonal antibodies that preferentially recognized the transition state or bound Aβ with high affinity were selected for expansion and further study.

<u>Propagation and purification of monoclonal antibodies</u>. Selected clones producing anti-Aβ antibodies and clones producing anti-receptor antibodies were injected into separate pristane-primed mice. Ascites were collected and the specific monoclonal antibodies isolated. Purification of antibodies from ascites was accomplished using a Protein A column or alternatively, antibodies were isolated from ascites fluid by $(NH_4)_2SO_4$ precipitation and passage over an S-300 column to obtain the 150 kDa immunoglobulin fraction. Monovalent Fab fragments were prepared and isolated by established methods. Their purity was evaluated by SDS-PAGE under reducing and non-reducing conditions. 50-100 mg of purified monoclonal antibody was routinely obtained from each ascites-bearing mouse.

<u>Further characterization of catalytic activity on Aβ substrates</u>. To fully define the hydrolytic properties of the isolated anti-transition state antibodies some very important controls can be run. First the ability to completely block catalytic antibody activity with the appropriate transition state peptide can be verified. This non-cleavable "inhibitor" should bind much more tightly to the antibody combining sites and thereby prevent substrate binding or cleavage. Substrate specificity can be further established by showing no cleavage of a sham Aβ peptide having a different amino acid sequence. The products of hydrolysis can also be fully characterized by HPLC, amino acid and mass spectral analysis. Control antibodies that are not

directed against the transition state $A\beta$ can be tested and confirmed to produce no catalysis. Finally, catalytic activity can be shown to reside in the purified Fab fragments of the anti-transition state antibody.

Purified anti-$A\beta$ antibodies dissolve preformed $A\beta$ aggregates. (Walker et al., *Soc. Neurosci. Abstr.* 21:257 (1995), Zlokovic, B.V. *Life Sciences* 59: 1483-1497 (1996)). $A\beta$ precipitates were formed and measured *in vitro* (Yankner et al., *Science* 250: 279-282 (1990), Kowall et al., *Proc. Natl. Acad. Sci.* 88: 7247-7251 (1991)). A radioactive assay was used to quickly screen the different monoclonal antibodies produced for an ability to dissolve preformed $A\beta$ aggregates. After adding ^{125}I-$A\beta$ to unlabeled soluble peptide, aggregates were formed by bringing the solution to pH 5 or by stirring it overnight in PBS. An aliquot of the labeled aggregate was incubated for 1 hr with either PBS, the 5A11 anti-$A\beta$ antibody or an equal amount of an irrelevant mouse antibody (7D3, anti-human transferrin receptor). After centrifugation, the level of radioactivity in the precipitate was measured.

Generation of vectorized anti-$A\beta$/anti-receptor bispecific antibodies. The anti-$A\beta$ antibodies were chemically coupled to anti-human transferrin receptor and anti-mouse transferrin receptor antibodies by different methods (Raso et al., *J. Biol. Chem.* 272: 27623-27628 (1997); Raso et al., Monoclonal antibodies as cell targeted carriers of covalently and non-covalently attached toxins. *In* Receptor mediated targeting of drugs, vol. 82. G. Gregoriadis, G. Post, J. Senior and A. Trouet, editors. NATO Advanced Studies Inst., New York. 119-138 (1984)). A rapid thioether linkage technique was used to form strictly bispecific hybrids using Traut's reagent and the heterobifunctional SMBP reagent. One component was sparingly substituted with thiol groups (SH). These readily reacted to form a thioether linkage

upon mixture with the maleimido-substituted (M) second component following the reaction:

$$Ab_A\text{-SH} + Ab_B\text{-M} \rightarrow Ab_A\text{-S-}Ab_B$$

Gel filtration of the reaction mixture on an S-300 column yielded the purified dimer which was 300 kDa and had two sites for binding Aβ plus two sites for attachment to transferrin receptors on brain capillary endothelial cells. Non-targeted control hybrids were formed by linking a nonspecific MOPC antibody to the anti-Aβ antibody. This hybrid antibody does bind Aβ, but, being non-reactive with transferrin receptors, should not cross the blood-brain barrier.

F(ab')$_2$ fragments of the two different antibody types can similarly be thioether-linked to form Fc-devoid reagents that cannot bind complement which might otherwise cause neurotoxic effects. These smaller bispecific hybrids (100 kDa) can be formed by reducing the intrinsic disulfides which link the heavy chains of F(ab')$_2$ fragments (Raso et al., *J. Immunol.* 125:2610-2616 (1980)). The thiols generated are stabilized and Ellman's reagent (E) is used to activate these groups on one of the components (Brennan et al., *Science* 229: 81-83 (1985)). Exclusively bispecific F(ab')$_2$ hybrids can be formed upon mixing the reduced Fab' with an activated Fab' having the alternate specificity according to the reaction:

$$Fab'_A\text{-SH} + Fab'_B\text{-SS-E} \rightarrow Fab'_A\text{-SS-}Fab'_B + \text{E-SH}$$

Purification on an S-200 column will isolate hybrids with one site for binding Aβ and one site for interaction with the target epitope on the brain capillary endothelial cells.

A similar approach can be used to make even smaller disulfide-linked single chain Fv heterobispecific dimers, Fv_A-SS-Fv_B (50 kDa), to cross the blood-brain barrier. Soluble Fvs can

be constructed to possess a carboxyl-terminal cysteine to facilitate the disulfide exchange shown in the reaction below, and create 50 kDa heterodimers exclusively:

$$Fv_A\text{-SH} + Fv_B\text{-SS-E} \rightarrow Fv_A\text{-SS-}Fv_B + \text{E-SH}$$

In side by side comparisons between whole antibody and either Fab' or Fv based bispecific reagents, the latter have proven to be moderately more effective on a molar basis for cell uptake via the transferrin receptor-mediated pathway (Raso et al., J. Biol. Chem. 272: 27623-27628 (1997)). Since these smaller constructs are monovalent for the cell-surface epitope, those findings dispel the notion that cross-linking of two surface receptors is necessary for the cellular uptake of immunocomplexes.

Functional assays for dual binding activity of bispecific antibodies. The capacity of the hybrid reagent to bind ^{125}I-Aβ was compared with that of the parent anti-Aβ antibody in a standard PEG binding assay (see Table 8 for binding assays).

The ability of the appropriate bispecific antibodies to attach to transferrin receptor bearing human or mouse cells was confirmed by cytofluorimetry. The bispecific antibody was reacted with transferrin receptor positive human or mouse cells and probed using either a rat IgG-specific or mouse IgG-specific fluorescent secondary antibody reagent.

Measurement of Aβ binding using ^{125}I-Aβ and a polyethylene glycol separation. To ensure bispecificity, hybrid reagents were tested for a capacity to mediate the attachment of ^{125}I-Aβ to receptor-bearing cells. Transferrin receptor positive cells were treated with the hybrid reagent, washed away unbound material and then exposed these cells to ^{125}I-A$\beta_{1\text{-}40}$. The cells were washed and the amount of cell-bound radioactivity was compared to control cells

which had been identically prepared except that pretreatment with bispecific antibody was omitted.

Capillary depletion. The bispecific antibody was labeled with ^{125}I and injected i.v. into normal mice. After different lengths of time the mice were sacrificed and the amount of ^{125}I-bispecific antibody that crossed the blood-brain barrier and entered the brain was gauged by a mouse capillary depletion method (Friden et al., *J. Pharm. Exper. Ther.* 278:1491-1498 (1996); Triguero et al., *J. Neurochem.* 54: 1882-1888 (1990)). The amount of vectorized bispecific antibody found in the brain parenchyma or brain capillary fractions was measured following differential density centrifugation of the brain homogenate. These values were plotted as a function of time after i.v. injection. Progressive passage from capillaries into the parenchyma indicates active transcytosis across the blood-brain barrier.

Immunoscintigraphy. A non-invasive method for monitoring intracerebral delivery process which involves visualizing the entry of a radiolabeled bispecific antibody into the brain of live mice, can also be used. Radiolabeled vectorized bispecific antibody (^{125}I-R17/5A11) or a non-vectorized control bispecific antibody were administered to separate mice. Sequential brain images were accumulated at 1, 6, 24 and 48 hours following i.v. administration of the ^{125}I-labeled bispecific antibody probes. The animals were chemically immobilized during exposure using ketamine/xylazine anesthesia. This imaging technology could be very useful for determining if circulating anti-Aβ antibodies will prevent i.v. administered ^{125}I-Aβ from entering the brain. Digital scintigraphy data was quantified using standards and the integration functions provided in the analysis software.

CLAIMS is characterized by the ability to

1. An antibody which catalyzes hydrolysis of β-amyloid at a predetermined amide linkage.

2. The antibody of Claim 1 which catalyzes hydrolysis of the amide linkage between residues X 39 and Y 40 of β-amyloid.

[VR: THE APPROPRIATE RESIDUES WILL BE FILLED IN FOR EACH OF THE DIFFERENT CATALYTIC ANTIBODIES] 40-41 41-42 19-20 20-21 38-39

3. The antibody of Claim 1 which preferentially binds a transition state analog which mimics the transition state adopted by β-amyloid during hydrolysis at a predetermined amide linkage, and also binds to natural β-amyloid with sufficient affinity to detect using an ELISA.

4. The antibody of Claim 1 which preferentially binds a transition state analog which mimics the transition state adopted by β-amyloid during hydrolysis at a predetermined amide linkage, and does not bind natural β-amyloid with sufficient affinity to detect using an ELISA.

5. A vectorized antibody which is characterized by the ability to cross the blood brain barrier and the ability to catalyze the hydrolysis of β-amyloid at a predetermined amide linkage.

6. The vectorized antibody of Claim 5 which is a bispecific antibody.

7. The vectorized antibody of Claim 6 which has a first specificity for the transferrin receptor and a second specificity for a transition state adopted by β-amyloid during hydrolysis.

8. The vectorized antibody of Claim 7 which catalyzes hydrolysis of β-amyloid between residues X and Y.

[VR: THE APPROPRIATE RESIDUES WILL BE FILLED IN FOR EACH OF THE DIFFERENT CATALYTIC ANTIBODIES]

9. A method for sequestering free β-amyloid in the bloodstream of an animal, comprising the steps:
   a) providing antibodies specific for β-amyloid; and
   b) intravenously administering the antibodies to the animal in an amount sufficient to increase retention of β-amyloid in the circulation.

10. A method for sequestering free β-amyloid in the bloodstream of an animal, comprising the steps:
    a) providing an antigen comprised of an epitope which is present on β-amyloid endogenous to the animal; and
    b) immunizing the animal with the antigen of step a) under conditions appropriate for the generation of antibodies which bind endogenous β-amyloid.

11. A method for reducing levels of β-amyloid in the brain of an animal, comprising the steps:
    a) providing antibodies specific for β-amyloid endogenous to the animal; and
    b) intravenously administering the antibodies to the animal in an amount sufficient to increase retention of β-amyloid in the circulation of the animal.

12. The method of Claim 11 wherein the antibodies specific for β-amyloid are catalytic antibodies which catalyze hydrolysis of β-amyloid at a predetermined amide linkage.

13. The method of Claim 11 wherein the antibodies are monoclonal.

14. The method of Claim 11 wherein the antibodies are polyclonal.

15. The method of Claim 11 wherein the antibodies specifically recognize epitopes on the C-terminus of β-amyloid$_{1-43}$.

16. A method for reducing levels of β-amyloid in the brain of an animal, comprising the steps:
   a) providing an antigen comprised of an epitope which is present on β-amyloid endogenous to the animal; and
   b) immunizing the animal with the antigen of step a) under conditions appropriate for the generation of antibodies which bind endogenous β-amyloid.

17. The method of Claim 16 wherein the antigen is a transition state analog which mimics the transition state adopted by β-amyloid during hydrolysis at a predetermined amide linkage.

18. The method of Claim 16 wherein the antigen is ....

19. The method of Claim 17 wherein the antibodies generated have a higher affinity for the transition state analog than for natural β-amyloid.

20. The method of Claim 17 wherein the antibodies generated catalyze hydrolysis of endogenous β-amyloid.

21. A method for preventing the formation of amyloid plaques in the brain of an animal, comprising the steps:
   a) providing an antigen comprised of an epitope which is present on β-amyloid endogenous to the animal; and
   b) immunizing the animal with the antigen of step a) under conditions appropriate for the generation of antibodies which bind endogenous β-amyloid.

22. The method of Claim 21 wherein the antigen is a transition state analog which mimics the transition state adopted by β-amyloid during hydrolysis at a predetermined amide linkage.

23. A method for reducing levels of circulating β-amyloid in an animal, comprising the steps:
    a) providing an antigen comprised of an epitope which is a mimic of a predetermined hydrolysis transition state of a β-amyloid polypeptide endogenous to the animal; and
    b) immunizing the animal with the antigen of step a) under conditions appropriate for the generation of antibodies to the β-amyloid hydrolysis transition state.

24. A method for reducing levels of circulating β-amyloid in an animal, comprising the steps:
    a) providing antibodies which catalyze the hydrolysis of β-amyloid endogenous to the animal; and
    b) intravenously administering the antibodies to the animal.

25. A method for preventing the formation of amyloid plaques in the brain of an animal, comprising the steps:
    a) providing antibodies which catalyze hydrolysis of β-amyloid produced by the animal at a predetermined amide linkage; and
    b) administering the antibodies to the animal in an amount sufficient to cause a significant reduction in β-amyloid levels in the blood of the animal.

26. A method for reducing levels of β-amyloid in the brain of an animal, comprising the steps:
    a) providing vectorized bispecific antibodies competent to transcytose across the blood brain barrier, which catalyze hydrolysis of β-amyloid of the animal at a

predetermined amide linkage; and

b) intravenously administering the antibodies to the animal.

27. The method of Claim 26 wherein the vectorized bispecific antibodies specifically bind the transferrin receptor.

28. The method of Claim 26 wherein the vectorized bispecific antibodies catalyze hydrolysis of the amide linkage between residues X and Y of β-amyloid.

[VR: THE APPROPRIATE RESIDUES WILL BE FILLED IN FOR EACH OF THE DIFFERENT CATALYTIC ANTIBODIES]

29. A method for disaggregating amyloid plaques present in the brain of an animal comprising the steps:

a) providing vectorized bispecific antibodies competent to transcytose across the blood brain barrier, which catalyze hydrolysis of β-amyloid produced by the animal at a predetermined amide linkage; and

b) intravenously administering the antibodies to the animal in an amount sufficient to cause significant reduction in β-amyloid levels in the brain of the animal.

30. A method for disaggregating amyloid plaques present in the brain of an animal, comprising the steps:

a) providing antibodies which catalyze hydrolysis of β-amyloid produced by the animal at a predetermined amide linkage; and

b) administering the antibodies to the animal.

31. The method of Claim 30 wherein the antibodies are bispecific vectorized antibodies competent for transcytosis across the blood-brain barrier.

32. A method for generating antibodies which catalyze hydrolysis of a protein or polypeptide comprising the steps:
    a) providing an antigen, the antigen being comprised of an epitope which has a statine analog which mimics the conformation of a predetermined hydrolysis transition state of the polypeptide;
    b) immunizing an animal with the antigen under conditions appropriate for the generation of antibodies to the hydrolysis transition state.

33. The method of Claim 32 wherein the protein is β-amyloid.

34. A method for generating antibodies which catalyze hydrolysis of a protein or polypeptide comprising the steps:
    a) providing an antigen, the antigen being comprised of an epitope which has a reduced peptide bond analog which mimics the conformation of a predetermined hydrolysis transition state of the polypeptide;
    b) immunizing an animal with the antigen under conditions appropriate for the generation of antibodies to the hydrolysis transition state.

35. The method of Claim 34 wherein the protein is β-amyloid.

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(207) 363-0558

June 30, 1999
Client No. BBRI-2004M

Boston Biomedical Research Institute
P.O. Box 981
Douglas MA 01516

Attn: Ms. Pamela Torpey

B-Amyloid Peptide

	Previous Balance			$9,765.22

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SYH	Incorporating inventor comments into Application; conferring with inventor (SYH, VR) re: his comments, suggestions, and new data submitted; preparing Application for filing.	100.00	3.50	350.00
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Expenses

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06/16/99	Photocopy charges	20.00
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June 30, 1999
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B-Amyloid Peptide

Balance Due $9,276.18

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=========

Human Compatible Vaccines for Treating Alzheimer's Disease

The concept of using the full-length 40-43 amino acid residue beta-amyloid peptide or shorter peptides derived from beta-amyloid as vaccines for treating or preventing Alzheimer's Disease was developed in my laboratory. The vaccines I used for preclinical studies in a transgenic mouse model of the disease were comprised of these beta-amyloid peptides chemically linked to Keyhole Limpet Hemacyanin and, to elicit an adequate immune response in the animals, Freunds complete or incomplete adjuvants were administered along with each antigen. Since this formulation is not suitable for use in humans, I have designed an alternative approach which uses genetic engineering to incorporate an appropriate beta-amyloid sequence into an antigenic protein. This new fusion antigen can then be administered with alum, the only currently licensed adjuvant for use in humans.

The best exemplification of related technology was the incorporation of various foreign peptide epitopes into the hepatitis B virus nucleocapsid antigen (1-4). These genetic constructs were introduced as vectors into bacteria for high-level expression of the different fusion polypeptide antigens. I have similarly designed genetic constructs that include the genetic code for either the full-length 40 amino acid sequence of beta-amyloid (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA) or the code for shorter epitopes derived from that sequence into the internal site of the hepatitis B virus nucleocapsid antigen (4). These new beta-amyloid antigens should elicit a high titered antibody and cellular response when used with alum as an adjuvant. Antigens possessing the smaller epitopes can be injected individually or in combination as part of a vaccine cocktail for treating or preventing Alzheimer's Disease.

1. Schodel, F., and H. Will. 1989. Construction of a plasmid for expression of foreign epitopes as fusion proteins with subunt B of Escherichia coli heat-labile enterotoxin. *Infection and Immunity* 57:1347-1350.

2. Schodel, F., A.M. Moriarty, D.L. Peterson, J. Zheng, J.L. Hughes, H. Will, D.J. Leturcq, J.S. McGee, and D.R. Milich. 1992. The position of heterologous epitopes inserted in Hepatitis B virus core particles determines their immunogenicity. *J. of Virology* 66:106-114.

3. Schodel, F., R. Wirtz, D. Peterson, J. Hughes, R. Warren, J. Sadoff, and D. Milich. 1994. Immunity to malaria elicited by hybrid Hepatitis B virus core particles carrying circumsporozoite. *J. of Experimental Medicine* 180:1037-1046.

4. Schodel, F., D. Peterson, J. Hughes, R. Wirtz, and D. Milich. 1996. Hybrid hepatitis B virus core antigen as a vaccine carrier moiety: I. Presentation of foreign epitopes. *J. of Biotechnol.* 44:91-96.

Read and understood. July 26, 1999

S Lehrer

Victor Rosso
7/26/99

Thomas J. McQuaid 7/26/99

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AA

Form Approved Through 9/30/97
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Public Health Service

Grant Application

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Type	Activity	Number
Review Group	Formerly	
Council/Board (Month, Year)		Date Received

1. TITLE OF PROJECT *(Do not exceed 56 characters, including spaces and punctuation.)*
IMMUNOTHERAPEUTIC AGENTS TO TREAT ALZHEIMER'S DISEASE

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT [ ] NO [X] YES *(If "Yes," state number and title)*
Number: PAS-99-034 Title: DRUG DISCOVERY FOR THE TREATMENT OF ALZHEIMER'S DISEASE

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR

3a. NAME *(Last, first, middle)*: RASO, VICTOR A.
3b. DEGREE(S): PH.D.
3c. SOCIAL SECURITY NO.:
3d. POSITION TITLE: SENIOR SCIENTIST
3e. MAILING ADDRESS *(Street, city, state, zip code)*:
BOSTON BIOMEDICAL RESEARCH INST.
20 STANIFORD STREET
BOSTON, MA 02114-2500
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TEL: (617) 912-0316
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4. HUMAN SUBJECTS [X] No [ ] Yes
4a. If "Yes," Exemption no. or IRB approval date [ ] Full IRB or [ ] Expedited Review
4b. Assurance of compliance no.

5. VERTEBRATE ANIMALS [ ] No [X] Yes
5a. If "Yes," IACUC approval date: 02/02/96
5b. Animal welfare assurance no.: A3177-01

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year-MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)
12/01/99	11/30/04	175,428	302,251	928,650	1,615,564

9. APPLICANT ORGANIZATION
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Address
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20 STANIFORD STREET
BOSTON, MA 02114-2500

10. TYPE OF ORGANIZATION
Public: → [ ] Federal [ ] State [ ] Local
Private: → [X] Private Nonprofit
Forprofit: → [ ] General [ ] Small Business

11. ORGANIZATIONAL COMPONENT CODE: 60

12. ENTITY IDENTIFICATION NUMBER: 1042451939A1
Congressional District: 9

13. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
Name: THOMAS J. MCQUAID
Title: ASSISTANT DIRECTOR
Address: BOSTON BIOMEDICAL RESEARCH INST.
20 STANIFORD STREET
BOSTON, MA 02114-2500
Telephone: (617) 912-0301
FAX: (617) 912-0335
E-Mail Address: MCQUAID@BBRI.ORG

14. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name: KATHLEEN G. MORGAN, PH.D
Title: DIRECTOR
Address: BOSTON BIOMEDICAL RESEARCH INST.
20 STANIFORD STREET
BOSTON, MA 02114-2500
Phone: (617) 912-0330
FAX: (617) 227-6053
E-Mail Address: MORGAN@BBRI.ORG

15. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PI/PD NAMED IN 3a. *(In ink. "Per" signature not acceptable.)*
Victor Raso
DATE: 1-19-99

16. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 14. *(In ink. "Per" signature not acceptable.)*
KG Morgan
DATE: 1/20/99

OMB No. 0925-0195
Expiration Date 4/30/01

Department of Health and Human Services
Public Health Service
Small Business Innovation Research Program
Phase II Grant Application
Follow instructions carefully.

Leave blank — for PHS use only.		
Type	Activity	Number
Review Group		Formerly
Council Board *(Month, year)*		Date Received

1a. TITLE OF APPLICATION *(Do not exceed 56 typewriter spaces)*
IMMUNOTHERAPY OF ALZHEIMER'S DISEASE

1b. Phase I Grant No.
1R43AG15746

2. PRINCIPAL INVESTIGATOR ☐ New Investigator

2a. NAME *(Last, first, middle)*
Raso, Victor

2b. DEGREE(S)
B.S. Ph.D.

2c. SOCIAL SECURITY NO. Provide on Personal Data Page

2d. POSITION TITLE
Senior Scientist

2e. MAILING ADDRESS *(Street, city, state, zip code)*
Boston Biomedical Res. Institute
20 Staniford Street
Boston, MA 02114

2f. TELEPHONE AND FAX *(Area code, number, and extension)*
TEL: (617) 912-0316
FAX: (617) 912-0308

BITNET/INTERNET Address:

3. HUMAN SUBJECTS
[X] NO
☐ YES

3a. If "yes," Exemption no. ☐ or IRB approval date ☐ < ☐ Full IRB or ☐ Expedited Review

3b. Assurance of compliance no.

4. VERTEBRATE ANIMALS
☐ NO
[X] YES

4a. If "Yes," IACUC approval date
6/7/99

4b. Animal welfare assurance no.
A3177-02

5. DATES OF ENTIRE PROPOSED PHASE II PERIOD
From: 03/01/00
Through: 02/28/02

6. COSTS REQUESTED FOR FIRST 12-MONTH BUDGET PERIOD
6a. Direct Costs $ 353,349
6b. Total Costs $ 378,083

7. COSTS REQUESTED FOR ENTIRE PROPOSED PHASE II PERIOD
7a. Direct Costs $682,260
7b. Total Costs $ 730,018

8. PERFORMANCE SITES *(Organizations and addresses)*
Boston Biomedical Res. Institute
20 Staniford Street
Boston, MA 02114

9. APPLICANT ORGANIZATION *(Name and address of applicant small business concern)*
Boston Biotechnology Corporation
20 Staniford Street
Boston, MA 02114

10. ENTITY IDENTIFICATION NUMBER
1042766443A1

Congressional District
9

11. INVENTIONS AND PATENTS
☐ NO [X] YES If "Yes," < ☐ Previously reported OR [X] Not previously reported

12. SMALL BUSINESS CERTIFICATION
[X] Small Business Concern ☐ Women-owned
☐ Socially and Economically Disadvantaged

13. NOTICE OF PROPRIETARY INFORMATION: The information identified by asterisks(*) on pages ____________ of this application constitutes trade secrets or information that is commercial or financial and confidential or privileged. It is furnished to the Government in confidence with the understanding that such information shall be used or disclosed only for evaluation of this application, provided that, if a grant is awarded as a result of or in connection with the submission of this application, the Government shall have the right to use or disclose the information herein to the extent provided by law. This restriction does not limit the Government's right to use the information if it is obtained without restriction from another source.

14. DISCLOSURE PERMISSION STATEMENT: If this application does not result in an award, is the Government permitted to disclose the title only of your proposed project, and the name, address, and telephone number of the official signing for the applicant organization, to organizations that may be interested in contacting you for further information or possible investment? ☐ YES [X] NO

15. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name: Thomas McQuaid
Title: Assistant Treasurer
Address: 20 Staniford Street
Boston, MA 02114

Telephone (617) 912-0301
FAX (617) 912-0335
BITNET/INTERNET Address.

B000187

16. PRINCIPAL INVESTIGATOR ASSURANCE: I certify that the statements herein are true, complete, and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PERSON NAMED IN 2a
(In ink. "Per" signature not acceptable.)
Victor Raso

DATE
8/13/99

17. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete, and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF PERSON NAMED IN 15
(In ink. "Per" signature not acceptable.)
Thomas J McQuaid

DATE
8/13/99

OMB No. 0925-0195
Expiration Date 2/28/98

Department of Health and Human Services
Public Health Service

Small Business Innovation Research Program

Phase I Grant Application

Follow instructions carefully.

Leave blank — for PHS use only.		
Type	Activity	Number
Review Group		Formerly
Council Board *(Month, year)*		Date Received

1. TITLE OF APPLICATION *(Do not exceed 56 typewriter spaces)*
CEREBRAL ANTIBODY DELIVERY TO TREAT ALZHEIMER'S DISEASE

2. SOLICITATION NO. PHS 97-2

3. PRINCIPAL INVESTIGATOR ☐ New Investigator

3a. NAME *(Last, first, middle)*
RASO, VICTOR A.

3b. DEGREE(S) B.S. | Ph.D. |

3c. SOCIAL SECURITY NO. Provide on Personal Data Page

3d. POSITION TITLE
SENIOR SCIENTIST

3e. MAILING ADDRESS *(Street, city, state, zip code)*
BOSTON BIOMEDICAL RES INST.
20 STANIFORD STREET
BOSTON, MA 02114

BITNET/INTERNET Address:

3f. TELEPHONE AND FAX *(Area code, number, and extension)*
TEL: (617) 742-2010, x316
FAX: (617) 523-6649

4. HUMAN SUBJECTS
[X] NO
☐ YES

4a. If "yes," Exemption no. ☐ or IRB approval date ☐ ☐ Full IRB or ☐ Expedited Review

4b. Assurance of compliance no.

5. VERTEBRATE ANIMALS
☐ NO
[X] YES

5a. If "Yes," IACUC approval date: 1/2/97

5b. Animal welfare assurance no.: A3177

6. DATES OF PROJECT PERIOD
From: 7/1/98 Through: 1/31/99

7. COSTS REQUESTED
7a. Direct Costs $ 100,000
7b. Total Costs $ 100,000

8. PERFORMANCE SITES *(Organizations and addresses)*
BOSTON BIOMEDICAL RES. INST.
20 STANIFORD STREET
BOSTON, MA 02114

9. APPLICANT ORGANIZATION *(Name and address of applicant small business concern)*
BOSTON BIOTECHNOLOGY CORP.
20 STANIFORD STREET
BOSTON, MA 02114

10. ENTITY IDENTIFICATION NUMBER 1042766443A1
Congressional District 9

11. SMALL BUSINESS CERTIFICATION
[X] Small Business Concern ☐ Women-owned
☐ Socially and Economically Disadvantaged

12. NOTICE OF PROPRIETARY INFORMATION: The information identified by asterisks(*) on pages ________ of this application constitutes trade secrets or information that is commercial or financial and confidential or privileged. It is furnished to the Government in confidence with the understanding that such information shall be used or disclosed only for evaluation of this application, provided that, if a grant is awarded as a result of or in connection with the submission of this application, the Government shall have the right to use or disclose the information herein to the extent provided by law. This restriction does not limit the Govemment's right to use the information if it is obtained without restriction from another source.

13. DISCLOSURE PERMISSION STATEMENT: If this application does not result in an award, is the Government permitted to disclose the title only of your proposed project, and the name, address, and telephone number of the official signing for the applicant organization, to organizations that may be interested in contacting you for further information or possible investment? [X] YES ☐ NO

14. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name: Vincent F. Raso
Title: Treasurer
Address: Boston Biotechnology Corp.
20 Staniford Street
Boston, MA 02114

Telephone: (617) 742-2010, x301
FAX: (617) 523-6649
BITNET/INTERNET Address:

15. PRINCIPAL INVESTIGATOR ASSURANCE: I certify that the statements herein are true, complete, and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PERSON NAMED IN 3a *(In ink. "Per" signature not acceptable.)*
Victor Raso
DATE 12/11/97

16. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete, and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF PERSON NAMED IN 14 *(In ink. "Per" signature not acceptable.)*
Vincent F. Raso
DATE 12/11/97

AA

Form Approved Through 9/30/97
OMB No. 0925-0001

Department of Health and Human Services
Public Health Service

Grant Application

Follow instructions carefully.
Do not exceed character length restrictions indicated on sample.

LEAVE BLANK-FOR PHS USE ONLY.		
Type	Activity	Number
Review Group	Formerly	
Council/Board *(Month, Year)*		Date Received

1. TITLE OF PROJECT *(Do not exceed 56 characters, including spaces and punctuation.)*
CEREBRAL DELIVERY OF VECTORIZED ANTI-ß-AMYLOID ANTIBODY

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT ☐ NO ☒ YES *(If "Yes," state number and title)*
Number: Title: PREPARED IN RESPONSE TO NIA AND NINDS ANNOUNCEMENT--ALZHEIMER'S DISEASE

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR

3a. NAME *(Last, first, middle)*: RASO, VICTOR A.

3b. DEGREE(S): PH.D.

3c. SOCIAL SECURITY NO.:

3d. POSITION TITLE: SENIOR SCIENTIST

3e. MAILING ADDRESS *(Street, city, state, zip code)*
BOSTON BIOMEDICAL RESEARCH INST.
20 STANIFORD STREET
BOSTON, MA 02114-2500

E-MAIL ADDRESS: RASO@BBRI.HARVARD.EDU

3f. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT

3g. MAJOR SUBDIVISION

3h. TELEPHONE AND FAX *(Area code, number and extension)*
TEL: (617) 912-0316
FAX: (617) 912-0308

4. HUMAN SUBJECTS ☒ No ☐ Yes

4a. If "Yes," Exemption no. or IRB approval date ☐ Full IRB or ☐ Expedited Review

4b. Assurance of compliance no.

5. VERTEBRATE ANIMALS ☐ No ☒ Yes

5a. If "Yes," IACUC approval date: 02/02/96

5b. Animal welfare assurance no.: A3177-01

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year-MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)
12/01/98	11/30/02	$198,885	$348,944	$829,005	$1,466,226

9. APPLICANT ORGANIZATION
Name
Address
BOSTON BIOMEDICAL RESEARCH INST.
20 STANIFORD STREET
BOSTON, MA 02114-2500

10. TYPE OF ORGANIZATION
Public: → ☐ Federal ☐ State ☐ Local
Private: → ☒ Private Nonprofit
Forprofit: → ☐ General ☐ Small Business

11. ORGANIZATIONAL COMPONENT CODE: 60

12. ENTITY IDENTIFICATION NUMBER: 1042451939A1

Congressional District: 9

13. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
Name: THOMAS J. MCQUAID
Title: ASSISTANT DIRECTOR
Address: BOSTON BIOMEDICAL RESEARCH INST.
20 STANIFORD STREET
BOSTON, MA 02114-2500
Telephone: (617) 912-0301
FAX: (617) 912-0335
E-Mail Address: MCQUAID@BBRI.HARVARD.EDU

14. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name: KATHLEEN G. MORGAN, PH.D
Title: DIRECTOR
Address: BOSTON BIOMEDICAL RESEARCH INST.
20 STANIFORD STREET
BOSTON, MA 02114-2500
Phone: (617) 912-0330
FAX: (617) 227-6053
E-Mail Address: MORGAN@BBRI.HARVARD.EDU

15. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE:
I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PI/PD NAMED IN 3a. *(In ink. "Per" signature not acceptable.)*
Victor Raso

DATE: 1/26/98

16. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE:
I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 14. *(In ink. "Per" signature not acceptable.)*
KGMorgan

DATE: 1-26-98

Department of Health and Human Services
Public Health Service

Small Business Innovation Research Program

Phase I Grant Application

Follow instructions carefully.

Leave blank — for PHS use only.		
Type	Activity	Number
Review Group		Formerly
Council Board *(Month, year)*		Date Received

1. TITLE OF APPLICATION *(Do not exceed 56 typewriter spaces)*
~~CATALYTIC ANTIBODIES TO INACTIVATE β-AMYLOID~~

2. SOLICITATION NO. PHS 97-2

3. PRINCIPAL INVESTIGATOR ☐ New Investigator

3a. NAME *(Last, first, middle)*: RASO, VICTOR A.

3b. DEGREE(S): B.S. | Ph.D

3c. SOCIAL SECURITY NO. *Provide on Personal Data Page*

3d. POSITION TITLE: SENIOR SCIENTIST

3e. MAILING ADDRESS *(Street, city, state, zip code)*
BOSTON BIOMEDICAL RES. INST.
20 STANIFORD STREET
BOSTON, MA 02114

BITNET/INTERNET Address:

3f. TELEPHONE AND FAX *(Area code, number, and extension)*
TEL: (617) 912-0316
FAX: (617) 912-0308

4. HUMAN SUBJECTS [X] NO ☐ YES
4a. If "yes," Exemption no. ☐ or IRB approval date ☐ < ☐ Full IRB or ☐ Expedited Review
4b. Assurance of compliance no.

5. VERTEBRATE ANIMALS ☐ NO [X] YES
5a. If "Yes," IACUC approval date: ~~1/2/97~~
5b. Animal welfare assurance no.: A3177-02

6. DATES OF PROJECT PERIOD
From: 10/01/9~~8~~ 9 Through: 03/31/~~98~~ 01

7. COSTS REQUESTED
7a. Direct Costs $400,000
7b. Total Costs $400,000

8. PERFORMANCE SITES *(Organizations and addresses)*
BOSTON BIOMEDICAL RES. INST.
20 STANIFORD STREET
BOSTON, MA 02114

9. APPLICANT ORGANIZATION *(Name and address of applicant small business concern)*
BOSTON BIOTECHNOLOGY CORP.
20 STANIFORD STREET
BOSTON, MA 02114

10. ENTITY IDENTIFICATION NUMBER: 1042766443A1
Congressional District: 9

11. SMALL BUSINESS CERTIFICATION
[X] Small Business Concern ☐ Women-owned
☐ Socially and Economically Disadvantaged

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13. DISCLOSURE PERMISSION STATEMENT: If this application does not result in an award, is the Government permitted to disclose the title only of your proposed project, and the name, address, and telephone number of the official signing for the applicant organization, to organizations that may be interested in contacting you for further information or possible investment? [X] YES ☐ NO

14. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name: ~~VINCENT F. RASO~~
Title: TREASURER
Address: 20 STANIFORD STREET
BOSTON, MA 02114
Telephone: (617) 912-0301
FAX: (617) 912-0335
BITNET/INTERNET Address:

15. PRINCIPAL INVESTIGATOR ASSURANCE: I certify that the statements herein are true, complete, and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PERSON NAMED IN 3a *(In ink. "Per" signature not acceptable.)*: Victor Raso
DATE: 4/13/98

16. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete, and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF PERSON NAMED IN 14 *(In ink. "Per" signature not acceptable.)*: Vincent J Raso
DATE: 4/13/98

AA

Form Approved Through 9/30/97
OMB No. 0925-0001

Department of Health and Human Services
Public Health Service

Grant Application

Follow instructions carefully.
Do not exceed character length restrictions indicated on sample.

LEAVE BLANK-FOR PHS USE ONLY.		
Type	Activity	Number
Review Group	Formerly	
Council/Board (Month, Year)		Date Received

1. TITLE OF PROJECT (*Do not exceed 56 characters, including spaces and punctuation.*)
VACCINE TO MODULATE SYSTEMIC β-AMYLOID LEVELS

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT ☐ NO ☒ YES (*If "Yes," state number and title*)
Number: PAR-98-047 Title: INNOVATIVE APPROACHES TO DEVELOPING NEW TECHNOLOGIES

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR

3a. NAME (*Last, first, middle*): RASO, VICTOR A.
3b. DEGREE(S): PH.D.
3c. SOCIAL SECURITY NO.:
3d. POSITION TITLE: SENIOR SCIENTIST
3e. MAILING ADDRESS (*Street, city, state, zip code*):
BOSTON BIOMEDICAL RESEARCH INST.
20 STANIFORD STREET
BOSTON, MA 02114-2500
3f. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT:
3g. MAJOR SUBDIVISION:
3h. TELEPHONE AND FAX (*Area code, number and extension*)
TEL: (617) 912-0316
FAX: (617) 912-0308
E-MAIL ADDRESS: RASO@BBRI.HARVARD.EDU

4. HUMAN SUBJECTS ☒ No ☐ Yes
4a. If "Yes," Exemption no. or IRB approval date ☐ Full IRB or ☐ Expedited Review
4b. Assurance of compliance no.

5. VERTEBRATE ANIMALS ☐ No ☒ Yes
5a. If "Yes," IACUC approval date: 02/02/96
5b. Animal welfare assurance no.: A3177-01

6. DATES OF PROPOSED PERIOD OF SUPPORT (month, day, year-MM/DD/YY) From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)
04/01/99	03/31/01	$75,000	$126,388	$150,000	$252,777

7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD
8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT

9. APPLICANT ORGANIZATION
Name
Address
BOSTON BIOMEDICAL RESEARCH INST.
20 STANIFORD STREET
BOSTON, MA 02114-2500

10. TYPE OF ORGANIZATION
Public: → ☐ Federal ☐ State ☐ Local
Private: → ☒ Private Nonprofit
Forprofit: → ☐ General ☐ Small Business

11. ORGANIZATIONAL COMPONENT CODE: 60

12. ENTITY IDENTIFICATION NUMBER: 1042451939A1
Congressional District: 9

13. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
Name: THOMAS J. MCQUAID
Title: ASSISTANT DIRECTOR
Address: BOSTON BIOMEDICAL RESEARCH INST.
20 STANIFORD STREET
BOSTON, MA 02114-2500
Telephone: (617) 912-0301
FAX: (617) 912-0335
E-Mail Address: MCQUAID@BBRI.HARVARD.EDU

14. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name: KATHLEEN G. MORGAN, PH.D
Title: DIRECTOR
Address: BOSTON BIOMEDICAL RESEARCH INST.
20 STANIFORD STREET
BOSTON, MA 02114-2500
Phone: (617) 912-0330
FAX: (617) 227-6053
E-Mail Address: MORGAN@BBRI.HARVARD.EDU

15. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PI/PD NAMED IN 3a. (*In ink. "Per" signature not acceptable.*)
Victor Raso
DATE: 5/20/98

16. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 14. (*In ink. "Per" signature not acceptable.*)
KG Morgan
DATE: 5/26/98

PHS 398 (Rev. 5/95)

B000740

Face Page

AA

AA

OMB No. 0925-0001

Department of Health and Human Services
Public Health Service

Grant Application

Follow instructions carefully.
Do not exceed character length restrictions indicated on sample.

LEAVE BLANK-FOR PHS USE ONLY.		
Type	Activity	Number
Review Group	Formerly	
Council/Board *(Month, Year)*		Date Received

1. TITLE OF PROJECT *(Do not exceed 56 characters, including spaces and punctuation.)*
PROBE TO VISUALIZE CEREBRAL β-AMYLOID PLAQUES

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT __ NO X YES *(If "Yes," state number and title)*
Number: PAR-98-012 Title: NEUROSCIENCES TECHNOLOGY DEVELOPMENT

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR

3a. NAME *(Last, first, middle)*: RASO, VICTOR A.
3b. DEGREE(S): PH.D.
3c. SOCIAL SECURITY NO.:
3d. POSITION TITLE: SENIOR SCIENTIST
3e. MAILING ADDRESS *(Street, city, state, zip code)*:
BOSTON BIOMEDICAL RESEARCH INST.
20 STANIFORD STREET
BOSTON, MA 02114-2500
E-MAIL ADDRESS: RASO@BBRI.HARVARD.EDU
3f. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT:
3g. MAJOR SUBDIVISION:
3h. TELEPHONE AND FAX *(Area code, number and extension)*
TEL: (617) 912-0316
FAX: (617) 912-0308

4. HUMAN SUBJECTS: [X] No [ ] Yes
4a. If "Yes," Exemption no. or IRB approval date [ ] Full IRB or Expedited Review
4b. Assurance of compliance no.

5. VERTEBRATE ANIMALS: [ ] No [X] Yes
5a. If "Yes," IACUC approval date: 02/02/96
5b. Animal welfare assurance no.: A3177-01

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year-MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)
04/01/99	03/31/01	$75,000	$126,388	$150,000	$252,777

9. APPLICANT ORGANIZATION
Name
Address
BOSTON BIOMEDICAL RESEARCH INST.
20 STANIFORD STREET
BOSTON, MA 02114-2500

10. TYPE OF ORGANIZATION
Public: → [ ] Federal [ ] State [ ] Local
Private: → [X] Private Nonprofit
Forprofit: → [ ] General [ ] Small Business

11. ORGANIZATIONAL COMPONENT CODE: 60

12. ENTITY IDENTIFICATION NUMBER: 1042451939A1
Congressional District: 9

13. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
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15. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PI/PD NAMED IN 3a. *(In ink. "Per" signature not acceptable.)* Victor Raso
DATE: 5/27/98

16. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 14. *(In ink. "Per" signature not acceptable.)* KG Morgan
DATE: 5/29/98

Fax sent 9/4/98

BOSTON BIOMEDICAL RESEARCH INSTITUTE

Victor A. Raso, Ph.D.

20 STANIFORD STREET, BOSTON, MASSACHUSETTS 02114
Area code 617 • 912-0316
Telefax 617 • 912-0308

Sept. 3, 1998

Mr. Nico Stanculescu
Research Grants Program of the
Alzheimer's Association

Dear Mr. Stanculescu,

I am sending you this letter-of-intent in advance of preparing an application for an Investigator Initiated Research Grant. The proposed research will be carried out at the Boston Biomedical Research Institute where I hold a senior scientist position. This project is aimed at the pre-clinical development of an catalytic antibody approach for the treatment and/or the prevention of Alzheimer's disease. It is titled "Cerebral Delivery of Vectorized Anti-ß-Amyloid Antibody" and encompasses three major specific aims.

- To Construct Vectorized Anti-ß-Amyloid/Anti-Receptor Bispecific Antibodies
- To Demonstrate that these Bifunctional Reagents Cross the Blood-Brain Barrier and Enter the Brain
- To Test the Vectorized Anti-ß-Amyloid/Anti-Receptor Bispecific Antibodies for the Disruption or Prevention of Cerebral Plaque Development in Transgenic Mice

Thank you for your kind consideration.

Very truly yours,

Victor Raso

Victor Raso, Ph.D.

B000393

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B. APPLICANT MANAGEMENT INFORMATION

Use Font Size 8 within the section B boxes for optimal performance

Single-Click on Table to Activate Text Box

Principal Investigator

Name:	Credentials:	Social Security No. (if non-US national Passport No.):	
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Grants Management Official

Name:	Credentials:		
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Institution Approval Requirements: Please Mark the Appropriate Box or Boxes with "XXX"

Human/Human Tissue Studies: IRB APPROVAL WILL BE/HAS BEEN OBTAINED	Animal/Animal Tissue Studies: IACUC APPROVAL WILL BE/HAS BEEN OBTAINED
	XXX

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Grant Information

Dollar Amount Requested:	Duration of Support	Start Date:
$180,000	3 years	04/01/98

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C. PROJECT ABSTRACT:

Enter a maximum of 21 lines of text using Font Size 12 in the box below.

We propose to develop and test several new, highly specific probes for imaging ß-amyloid plaques in the brain of Alzheimer's patients. These non-invasive, radiolabeled probes will be composed of two basic components. The first part is a monoclonal anti-ß-amyloid antibody that recognizes and selectively binds to the essential peptide constituent of plaques. In order to transport this anti-ß-amyloid antibody across the blood-brain barrier and into the brain, it will be coupled to an anti-transferrin receptor antibody. The resulting vectorized, bifunctional probe can bind to transferrin receptors which are abundant on the cerebral capillary endothelial. Subsequently, this receptor-bound bispecific antibody will be carried into the brain via transcytosis and, thus situated, can specifically accumulate on ß-amyloid plaques.

The ability of ^{125}I- or ^{99m}Tc-labeled vectorized probes to specifically target ß-amyloid plaques will be evaluated using an established colony of TS2576 transgenic mice. These animals express a mutant form of the human amyloid precursor protein and produce extracellular ß-amyloid peptide deposits in the brain. Brain scans will be taken at intervals following i.v. injection of the labeled probes so that the radioactivity can be localized and quantitated. Comparisons will be made between the distribution of probes in the brain of older plaque-bearing transgenic mice and younger control mice which have not yet developed cerebral amyloid deposits.

D. PROJECT KEYWORDS:

Enter below a maximum of 10 project description keywords separated by commas.

Keywords: ß-amyloid, bispecific antibody, vector, radiolabel, blood-brain barrier, ^{125}Iodine, ^{99m}Technetium

E. PROBLEM STATEMENT:

Enter a maximum of 42 lines of text using Font Size 12 in the box below and the continuation box on the next page.

Alzheimer's disease is a progressive and ultimately fatal form of dementia that affects a substantial portion of the elderly population. Effective treatment of this disease would be facilitated if we had the ability to detect the condition early, before irreversible damage has occured. We propose to design, construct and test radiolabeled probes that will specifically identify and quantitate one of the hallmarks of Alzheimer's disease, the abnormal accumulation of ß-amyloid peptide (Aß) into cerebral plaques. These imaging probes can also serve to track disease progression or its remission following treatment.

Diagnosis of Alzheimer's disease at autopsy relies on the presence of neuropathological brain lesions marked by a high density of senile plaques. These extracellular deposits are in the neo-cortex, hippocampus and amygdala as well as in the walls of the meningeal and cerebral blood vessels. The principal component of these plaques is a 39-43 residue ß-amyloid peptide. Each plaque contains ~ 20 fmole of this 4 kDa peptide (1). Apolipoprotein E and neurofibrillary tangles formed by the microtubule-associated tau protein are also often associated with Alzheimer's disease.

Aß is proteolytically cleaved from an integral membrane protein called the ß-amyloid precursor protein. The gene that codes for this protein in humans is found on chromosome 21 (2,3). While it is difficult to establish an absolute causal relationship between Aß or the plaques it forms and Alzheimer's disease, there is ample evidence to support the pathogenic role of Aß. Patients with Down's syndrome have an extra copy of the ß-amyloid precursor protein gene due to trisomy of chromosome 21 (2,3). They correspondingly develop an early-onset Alzheimer's disease neuropathology at 30-40 years of age. Moreover, familial Alzheimer's disease can result from mutations in the ß-amyloid precursor protein gene which fall within or adjacent to the Aß sequence (4).

Given the central role played by Aß in Alzheimer's disease, this definitive marker and the plaques it forms are obvious targets for an early detection system. Specific recognition of Aß in cerebral plaques might be accomplished by using monoclonal anti-Aß antibodies selected to tightly bind this peptide. However, antibodies in the peripheral circulation do not normally cross the blood-brain barrier. Blood-borne anti-Aß antibodies therefore, cannot reach Aß plaques in the central nervous system.

Fortunately, a universal method for the rapid, vector-mediated delivery of macromolecules across the blood-brain barrier has been devised (5-8). This novel system will be used to carry radiolabeled anti-Aß over the blood-brain barrier so that it comes into immediate contact with Aß plaques in brain (Fig. 1). An anti-transferrin receptor will be attached to the anti-Aß so that the new vectorized bifunctional construct

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PROBLEM STATEMENT - Page Two:

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can bind to receptors on the cerebral capillaries and cross into the brain by transcytosis. Areas of the brain with a high density of plaques will accumulate the radiolabeled probe and will become readily apparent when visualized by single-photon emission computed tomography (SPECT).

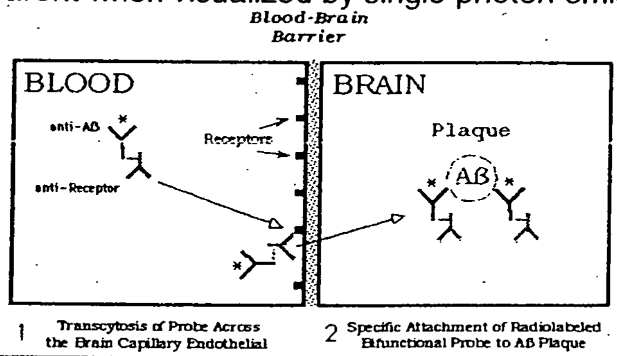

Fig. 1

End of Problem Statement

F. WORK PLAN:

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A vector moiety must be chemically or genetically attached to the anti-Aβ antibody to facilitate its delivery into the central nervous system. This vector component could be, for example, an anti-transferrin receptor or anti-insulin receptor antibody that binds to those receptors on the brain capillary endothelial cells (5-8) which make up the blood-brain barrier. The resulting bifunctional antibody (9-11) will attach to appropriate receptors on the luminal side of the vessel (Fig. 1). Once bound to the receptor, both components of the bispecific antibody will pass across the blood-brain barrier by the process of transcytosis. Anti-Aβ antibodies which have entered the brain can interact directly with Aβ plaques. It has been estimated that concentrations of macromolecules in the 10^{-8}-10^{-7}M range can be achieved in the brain using vector-mediated delivery via these brain capillary enriched protein target sites (12). Importantly, the vector appears safe since animals dosed daily for two weeks with an anti-transferrin receptor antibody showed no loss of integrity of the blood-brain barrier using radioactive sucrose (13).

Anti-Aβ Antibodies: The amino acid sequence of the 43 residue β-amyloid peptide is DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIAT ($A\beta_{1-43}$). Predicting precisely which site on the Aβ peptide will be best suited for

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WORK PLAN - Page Two:

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antibody-mediated labeling of cerebral plaques is difficult. Therefore we synthesized 3 key epitopes on the Aß 43-mer ($Aß_{1-17}$, $Aß_{10-25}$ and $Aß_{35-43}$) using standard automated Fmoc chemistry. A terminal Cys substitution was added to each so that these small peptides could be coupled to an antigenic, maleimide-activated carrier proteins such as Keyhole Limpet hemocyanin (KLH). These Aß antigens are being used to elicit peptide-specific monoclonal antibodies. The resulting panel of antibodies will then be screened by a variety of *in vitro* assays to identify desirable properties such as high affinity binding and the ability to react with Aß aggregates.

For example, separate ELISAs were used to screen and characterize hybridoma clones generated by the small carboxy-terminal peptide $Aß_{35-43}$ (Fig. 2). The monoclonal antibodies bound to the carboxy-terminal locus on each of these carrier-free

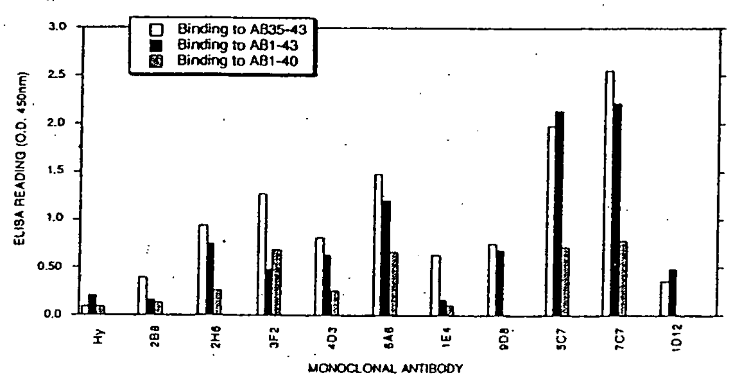

Fig. 2 ELISA Comparing Antibody Binding to $Aß_{35-43}$ and $Aß_{1-43}$ Versus $Aß_{1-40}$

Aß peptides adsorbed directly to the microtitre plate, confirming their anti-peptide specificity (Fig. 2). They did not react as strongly with a shortened, 40 amino acid residue version of Aß, showing their preference for the carboxy-terminus of Aß (Fig. 2). An ability to distinguish full-length $Aß_{1-43}$ may be important since this species is most pathogenic and is more prevalent in cerebral plaque deposits than shorter forms (14).

We radiolabeled $Aß_{1-40}$ and $Aß_{1-43}$ with ^{125}I and then separated the iodinated peptide from unlabeled material by (15). This probe was incubated with either purified anti-Aß antibodies or media taken from hybridoma clones producing anti-Aß antibodies. A polyethylene glycol separation method will be used to detect the amount of ^{125}I-$Aß_{1-43}$ bound to antibody. One of our purified anti-Aß monoclonal antibody bound 80 percent of

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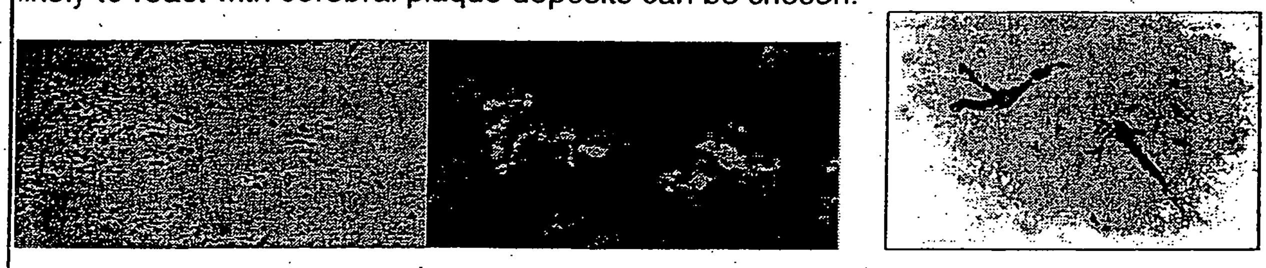
the added ^{125}I-Aß. This binding assay will allow us to quickly identify clones which produce high affinity anti-Aß antibodies.

The self-aggregation of synthetic Aß peptides leads to microscopic structures which resemble amyloid plaques in the brain and exhibit the same bright green fluorescence upon exposure to thioflavin T (Fig. 3A). Aß aggregates will be used to screen for the binding of ^{125}I-labeled anti-Aß monoclonal antibodies so that those most likely to react with cerebral plaque deposits can be chosen.



Phase Contrast A Thioflavin Fluorescence B

Fig. 3 Photomicrographs of (A) Self-Aggregated Aß Peptide and (B) Human Brain Capillaries Stained with the 7D3 Anti-TfR Antibody (16)

Vector for Transcytosis Across the Blood-brain Barrier: Anti-transferrin receptor antibodies (anti-TfR) are the primary vectors that we will deploy for delivery of anti-Aß antibodies into the brain. The 7D3 mouse monoclonal antibody developed in this lab, is specific for the human receptor and selectively immunostains cortical capillaries in normal human brain tissue (16) (Fig. 3B). Bispecific antibodies constructed with this 7D3 antibody and an anti-Aß antibody would be potentially useful for imaging in patients with Alzheimer's disease and possibly for preclinical trials in primates. For studies in the transgenic mouse model of Alzheimer's disease we have obtained an anti-mouse transferrin receptor monoclonal antibody produced the rat.

Synthesis and Characterization of Vectorized Bispecific Antibodies: The anti-Aß antibodies have been chemically coupled to anti-human transferrin receptor and anti-mouse transferrin receptor antibodies by different methods (9-11). We adopted a rapid thioether linkage technique to form strictly bispecific hybrids using Traut's reagent and the heterobifunctional SMBP reagent. One component was sparingly substituted with thiol groups (SH). These readily reacted to form a thioether linkage upon mixture with the maleimido-substituted (M) second component [Ab_A-SH + Ab_B-M $\rightarrow$ Ab_A-S-Ab_B]. $F(ab')_2$ fragments of the two different antibody types will be similarly thioether-linked to form small Fc-devoid reagents that cannot bind complement which might otherwise

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cause neurotoxic effects. Non-targeted control hybrids were formed by linking a nonspecific antibody to the anti-Aß antibody. This hybrid antibody will bind Aß, but, being non-reactive with transferrin receptors, should not cross the blood-brain barrier.

The ability of the bifunctional reagent to attach to transferrin receptor bearing human cells was confirmed by cytofluorimetry using an anti-mouse IgG probe (Fig. 4) and it also bound ^{125}I-Aß. Its dual specificity was clearly shown by its ability to simultaneously attach to cell membrane receptors and bind ^{125}I-Aß$_{1\text{-}40}$ (Table I).

Table I Bispecific Antibody-Mediated Binding of ^{125}I-Aß to Receptor-Positive Cells

Pretreatment of Cells	^{125}I-Aß Bound (cpm)
None	2,367
+ anti-Aß/anti-transferrin receptor	11,476

We coupled a rat monoclonal anti-mouse transferrin receptor antibody to a mouse monoclonal anti-Aß so that the entry of this new vectorized bispecific construct into brain could be monitored. The bispecific antibody was labeled with ^{125}I and injected i.v. into normal mice (no Aß plaques). After different times they were sacrificed and the amount of ^{125}I-bispecific antibody which crossed the blood-brain barrier and entered the brain was determined by a mouse capillary depletion method (Fig. 5)(8, 17). The time-dependent redistribution of radiolabeled bispecific antibody from the capillaries and into the parenchyma is consistent with its passage across the blood-brain barrier (17).

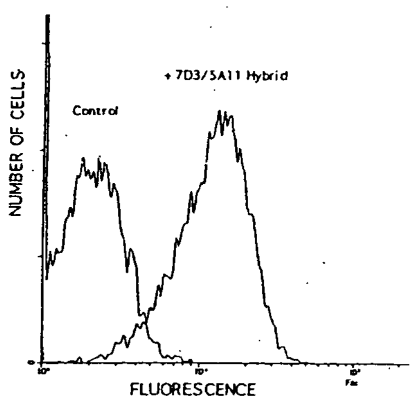

Fig. 4 Attachment of Bispecific Antibody to Receptor-Positive Cells

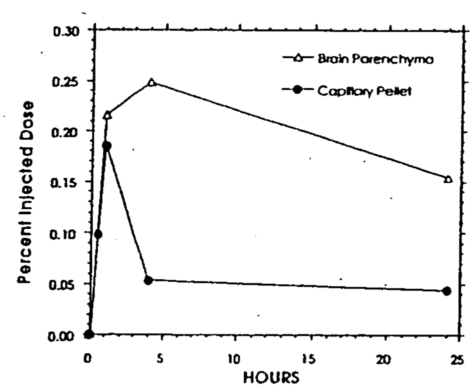

Fig. 5 Transcytosis of the Vectorized Bispecific Antibody into Brain

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An ability to follow the entry and accumulation of vectorized bispecific antibodies in the brain of live plaque-bearing transgenic mice (18) would assist in developing a system for intracerebral imaging of Alzheimer's patients. Therefore, we administered either the ^{125}I-labeled vectorized bispecific antibody or a ^{125}I-labeled non-vectorized control bispecific antibody to separate normal mice (no Aß plaques). Sequential brain images of the living mice were accumulated at 1, 6, 24 and 48 hours following i.v. administration of the ^{125}I-labeled bispecific antibody probes. When the vectorized agent was used, brain levels increased between 1 and 6 hrs and then declined to a lower level at 24 and 48hrs. Mice treated with the control displayed no increase between 1 and 6 hrs. Alternative radioactive labels such as ^{111}In or ^{99m}Tc will be attached to the vectorized bispecific antibody to compare with the results obtained with ^{125}I-labeled probes. Importantly, the digitital scintigraphy data can be easily quantified using standards and the integration functions provided in the analysis software. The central red (dark) region in the brain image shown below corresponds to ~1.5 x 10^5 cpm.

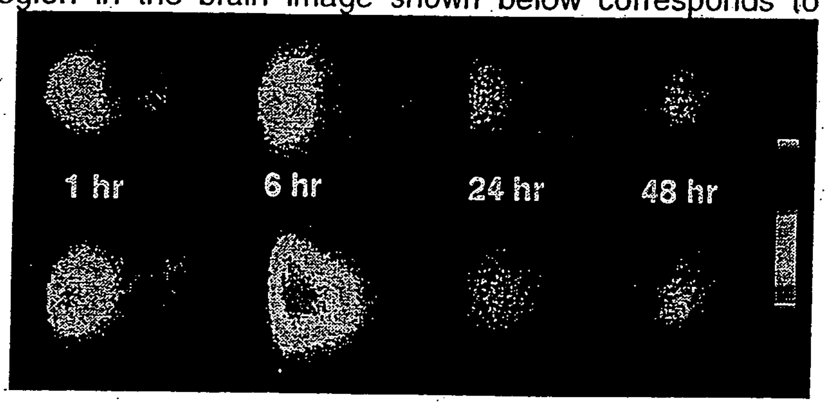

Fig. 6 Dorsal Aspect Brain Images of Mice (Nose to Right) Treated with Vectorized (bottom) or Control (top) Bispecific Antibody; Red in the Image Denotes Greater CPM

We will next examine older, plaque-bearing transgenic mice (8) to determine if a greater or more prolonged accumulation of the radiolabeled vectorized probe will result (Fig. 5 and 6). Smaller vectorized $F(ab')_2$ fragments also will be tested for improved intracerebral targeting. Our rational assembly of reagents, techniques and model system currently puts us in a unique position to establish the scientific basis for this novel and potentially high impact approach for the early detection of Alzheimer's disease using an Aß-specific brain imaging probe.

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WORK PLAN - Page Six:

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18. K. Hsiao, et al., Science **274**, 99-102 (1996)

End of Work Plan

G. APPLICANT INFORMATION:

Section 4.5.f Scientific, Technical and Academic Qualifications.

Enter a maximum of 42 lines of text using Font Size 12 (Arial) in the box below and the continuation box on the following page.

Principal Investigator: Victor A. Raso, Ph.D.

Professional Experience:

1973-1974	Associate in Pharmacology, Harvard Medical School
1973-1981	Research Associate, Division of Biochemical Pharmacology, Dana-Farber Cancer Institute
1981-1988	Assistant Professor of Pathology, Dana-Farber Cancer Institute, Harvard Medical School
1988-1989	Principal Scientist, Boston Biomed Res Inst., Dept. of Cell & Mol Bio
1989-	Senior Scientist, BBRI, Dept. of Cell & Molecular Biology

Publications (Partial):

1. Raso, V and Stollar, BD. Antibodies specific for conformationally distinct coenzyme-substrate transition state analogues. A fluorescence, N.M.R., circular dichroism and antibody study of N-(5-phosphopyridoxyl)-3'-amino-L-tyrosine. J. Amer. Chem. Soc. 1973; 95:1621.
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Scientific, Technical and Academic Qualifications - Page Two
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15. Raso, V. Intracellular targeting using bispecific antibodies. Meth. Mol. Med. in press

End of Scientific, Technical and Academic Qualifications

Section 4.5.2 and 4.5.3 List of Qualified Scientific and Technical Reviewers and List of Conflicts:

In the box below, serially enter the names of individuals (and their affiliation) with whom you are in conflict (*e.g., Joan Doe, MD (State Medical University), Joe Roe, Ph.D. (National University)*).

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REVIEWERS: ***Please provide at least 5 and no more than 15 individual contacts (Use Font Size 8)***

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Dr. Thomas Griffin	Oncology/Immunology, Hoffman-LaRoche, Inc., 340 Kingsland St, utley, NJ 07110-1199	(973) 562-3460	(973) 235-4044	tom.griffin@roche.com
Dr. D. J. Hnatowich	Dept. Med., U Massachusetts Med Center, Worcester, MA 01655	(508) 856-4256	(508) 856-4572	dhnatowich@banyon.ummed.edu
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Dr. R. E. Tanzi	Dept. Neurol., Massachusetts Gen. Hosp., Charlestown, MA 02129	(617) 726-6845	(617) 726-5736	tanzi@helix.mgh.harvard.edu

End of List of Qualified Reviewers and Conflicts

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H. AVAILABLE RESOURCES & BUDGET JUSTIFICATION:

Enter a maximum of 42 lines of text using Font Size 12 in the box below and the continuation box on the following page.

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Personnel: The P.I. will devote 20% time/effort to supervise the overall project and the scientific endeavors of the Research Technician, Christine Kearney (50% time/effort). The P.I. and Res. Tech. will take responsibility for the synthesis and evaluation of all peptides, their conjugation to antigenic carriers, as well as production of monoclonal antibodies and their characterization. They will synthesize the vectorized bispecific antibodies and verify their bifunctional activity. They will radiolabel the probes with ^{125}I, ^{111}In or ^{99m}Tc. They will determine if i.v. administered anti-Aß probes enter the brain of older transgenic mice and specifically localize within regions containing plaques.

Continue to next page.

AVAILABLE RESOURCES & BUDGET JUSTIFICATION - Page Two

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Other: Animal housing is requested for maintenance of the transgenic mice and mice used in the hybridoma fusions and for ascites production. Charges for analysis of bispecific antibodies using the cytofluorimetry facility are also included.

End of Available Resources & Budget Justification

KRS: *APPLICATION FORM FOR INDIVIDUAL RESEARCH PROJECT©*
Program Solicitation Number:
Application Number:
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Submittal Date:

I. COST ESTIMATE:

Categories	YEAR 1	YEAR 2	YEAR 3
Personnel:			
Salaries: List here each member of the team plus % of time (FTE) allocated to the project			
Victor Raso (20%)	20,080	20,080	20,080
Christine Kerney (50%)	13,060	13,060	13,060
Benefits: List here each member of the team plus % of time (FTE) allocated to the project			
Victor Raso (20%)	6,827	6,827	6,827
Christine Kerney (50%)	4,440	4,440	4,440
Lab and Research:			
Supplies Including Animals:	7,600	7,600	7,600
Computer Equipment & Services:	0	0	0
Consultants:			
Publication Costs:	300	300	300
Travel: (# $1000)	0	0	0
Subcontracts:	0	0	0
Other:	2,238	2,238	2,238
Total Direct Costs:	54,545	54,545	54,545
Indirect Costs:	5,455	5,455	5,455
Total Direct & Indirect Costs:	60,000	60,000	60,000
Cost Sharing:			

G. APPLICANT INFORMATION:

Section 4.5.f Scientific, Technical and Academic Qualifications.

Enter a maximum of 42 lines of text using Font Size 12 (Arial) in the box below and the continuation box on the following page.

Principal Investigator: Victor A. Raso, Ph.D.

Professional Experience:

1973-1974	Associate in Pharmacology, Harvard Medical School
1973-1981	Research Associate, Division of Biochemical Pharmacology, Dana-Farber Cancer Institute
1981-1988	Assistant Professor of Pathology, Dana-Farber Cancer Institute, Harvard Medical School
1988-1989	Principal Scientist, Boston Biomed Res Inst., Dept. of Cell & Mol Bio
1989-	Senior Scientist, BBRI, Dept. of Cell & Molecular Biology

Publications (Partial):

1. Raso, V and Stollar, BD. Antibodies specific for conformationally distinct coenzyme-substrate transition state analogues. A fluorescence, N.M.R., circular dichroism and antibody study of N-(5-phosphopyridoxyl)-3'-amino-L-tyrosine. J. Amer. Chem. Soc. 1973; 95:1621.
2. Raso V and Stollar BD. The antibody-enzyme analogy. Characterization of antibodies to phosphopyridoxyltyrosine derivatives. Biochem. 1975; 14: 584-591.
3. Raso V and Stollar BD. The antibody-enzyme analogy. Comparison of enzymes and antibodies specific for phosphopyridoxyltyrosine. Biochem. 1975; 14: 591-599.
4. Raso V and Griffin T. Specific cytotoxicity of a human immunoglobulin directed Fab'-ricin A chain conjugate. J Immunol. 1980; 125 :2610.
5. Raso V and Griffin T. Hybrid antibodies with dual specificity for the delivery of ricin to immunoglobulin bearing target cells. Cancer Res. 1981; 41:2073.
6. Raso V, Ritz, J. Basala M and Schlossman SF. A monoclonal antibody-ricin A chain conjugate which is selectively cytotoxic for cells bearing the common acute lymphoblastic leukemia antigen (CALLA). Cancer Res. 1982; 42:457.
7. Raso V. Antibody mediated delivery of toxin molecules to antigen bearing target cells. In: Moller, G, Ed. Immunological Reviews: Antibody carriers of drugs and toxins in tumor therapy. Copenhagen: Munksgaard, 1982: 93-117.
8. Raso V. and McGrath J. Diphtheria toxin cures athymic mice of human malignant mesothelioma. J. Natl. Cancer Inst. 1989; 81, 622-627.
9. Recht, L., Torres, C. O., Smith, T. W., Raso, V. A., and Griffin, T. W. (1990) Transferrin receptor in normal and neoplastic brain tissue: Implications for brain-tumor immunotherapy. *J Neurosurg* **72,** 941-945.
10. Recht LD, Griffin TW, Raso V, Salimi AR. Potent cytoxicity of an antihuman transferrin receptor-ricin A-chain immunotoxin on human glioma cells *in vitro.*

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Scientific, Technical and Academic Qualifications - Page Two
Continue Scientific, Technical and Academic Qualifications text in box below.

Cancer Res 1990; 50: 6696-6700.

11. Recht, L., Raso, V. Davis, R. and Salmonsen, R. Immunotoxin sensitivity of CHO cells expressing human transferrin receptors with differing internalization rates. Cancer Immunol. Immunotherpy. 1996; 42: 357-361
12. Raso, V. Immunotargeting Intracellular Compartments. Anal. Biochem. 1994; 222: 294-304.
13. Raso, V., Brown, M., McGrath, J., Liu, S. and Stafford, W. Antibodies capable of releasing diphtheria toxin in response to the low pH found in endosomes. J. Biol. Chem. 1997; 272: 27618-27622.
14. Raso, V., Brown, M. and McGrath, J. Intracellular targeting with low pH triggered bispecific antibodies. J. Biol. Chem. 1997; 272: 27623-27628.
15. Raso, V. Intracellular targeting using bispecific antibodies. Meth. Mol. Med. in press

End of Scientific, Technical and Academic Qualifications

Section 4.5.2 and 4.5.3 List of Qualified Scientific and Technical Reviewers and List of Conflicts:

In the box below, serially enter the names of individuals (and their affiliation) with whom you are in conflict (*e.g., Joan Doe, MD (State Medical University), Joe Roe, Ph.D. (National University)*).

Continue to next page.

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Cost Sharing:			

BOSTON BIOMEDICAL RESEARCH INSTITUTE

Victor A. Raso, Ph.D.

20 STANIFORD STREET, BOSTON, MASSACHUSETTS 02114
Area code 617 • 912-0316
Telefax 617 • 912-0308

Sept. 4, 1998

Mr. Nico Stanculescu
Research Grants Program of the
Alzheimer's Association

Dear Mr. Stanculescu,

I am sending you this letter-of-intent in advance of preparing an application for an Investigator Initiated Research Grant. The proposed research will be carried out at the Boston Biomedical Research Institute where I hold a senior scientist position. This project is aimed at the pre-clinical development of an catalytic antibody approach for the treatment and/or the prevention of Alzheimer's disease. It is titled "Catalytic Antibodies to Inactivate ß-Amyloid" and encompasses three major specific aims.

- To Construct Transition State ß-Amyloid Antigens
- To Produce Catalytic Anti-ß-Amyloid Monoclonal Antibodies
- To Test the Catalytic Anti-ß-Amyloid Monoclonal Antibodies for the Disruption or Prevention of Cerebral Plaque Development in Transgenic Mice

Thank you for your kind consideration.

Very truly yours,

Vic Raso

Victor Raso, Ph.D.

KRS: *APPLICATION FORM FOR INDIVIDUAL RESEARCH PROJECT*©
Application Number *(for office use only)*:
Project Title:

A. APPLICANT MANAGEMENT INFORMATION *(Instructions: ctrl+alt A)*

Principal Investigator

Name	Credentials	Social Security No. (if non-US national Passport No.)	
Victor A. Raso	Ph.D.	050-36-7225	
Address	**E-mail Address**	**Phone**	**FAX**
Boston Biomedical Research Institute 20 Staniford St. Boston, MA 02114	Raso@bbri.harvard.edu	617, 912-0316	617, 912-0308

Scientific Staff - Collaborators and Consultants

Name (Affiliation)	SS# (Passport #)	Name (Affiliation)	SS# (Passport #)

Fiscal Responsibility (business official who will have fiscal responsibility for this application)

Name	Credentials	Social Security No. (if non-US national Passport No.)	
Thomas J. McQuaid	MBA, MST, CPA	024-44-8367	
Address	**E-mail Address**	**Phone**	**FAX**
Boston Biomedical Research Institute 20 Staniford St. Boston, MA 02114	McQuaid@bbri.harvard.ed u	617, 912-0301	617, 912-0335

Institution Approval Requirements: Please Mark the Appropriate Box or Boxes with "XXX"

Human/Human Tissue Studies IRB APPROVAL WILL BE/HAS BEEN OBTAINED	Animal/Animal Tissue Studies IACUC APPROVAL WILL BE/HAS BEEN OBTAINED
	XXX

Grant Information

Dollar Amount Requested	Duration of Support	Start Date
$180,000	3 yrs.	04/01/99

Project Title

Catalytic Antibodies to Inactivate Beta-Amyloid

Competition (IIRG=Investigator Initiated Research Grant, PRG=Pilot RG, HAT=Hatfield RG, ZEN=Zenith RG, PIO=Pioneer RG)

IIRG

B. PROJECT ABSTRACT *(Instructions: ctrl+alt B)*

The aim of this project conducted by Dr. Victor Raso at the Boston Biomedical Research Institute is to develop catalytic antibodies for the treatment of Alzheimer's disease. Beta-amyloid and the plaques it forms are likely either the direct or indirect cause of Alzheimer's disease. Soluble beta-amyloid exists free in the blood and cerebrospinal fluid and eventually deposits as "insoluble" aggregates to produce cerebral plaques. Since the full-length, 43-residue beta-amyloid peptide initiates aggregation, catalytic antibodies that selectively cleave this 43-mer into harmless, non-amyloidogenic fragments could be therapeutically useful for Alzheimer's disease.

Transition state antigens will be designed to elicit catalytic antibodies with a capacity to cleave beta-amyloid peptides into non-amyloidogenic fragments. This irreversible modification could prevent the onset of Alzheimer's disease or impede its progression. Highly efficient, catalytic antibodies can permanently inactivate many target molecules rather than just acting stoichiometrically. Beta-amyloid transition state antigens might yield new clinical vaccines for combating Alzheimer's disease.

Transition state analogs of beta-amyloid have been synthesized for use as antigens to generate unique monoclonal antibodies. This panel of reagents will be screened for catalytic antibodies that can effectively destroy beta-amyloid. The catalytic antibodies will be tested therapeutically in a transgenic mouse model. They can be used systemically to greatly reduce circulating levels of beta-amyloid. Alternatively, the catalytic antibodies might be delivered into the brain via a vector-mediated transport system so that they can directly deplete amyloid deposits.

C. PROJECT KEYWORDS *(Instructions: ctrl+alt C)*

Project Keywords (Please choose from accompanying list where possible)	
Beta-amyloid	Proteases
Immunology	* Catalytic
Plaques	* Antibody
Transgenic Mice	* Vaccine
Enzymology	

D. PROBLEM STATEMENT *(Instructions: ctrl+alt D)*

Alzheimer's disease is a progressive and ultimately fatal form of dementia that affects a substantial portion of the elderly population. Definitive diagnosis at autopsy relies on the presence of neuropathological brain lesions marked by a high density of senile plaques. The principal component of these plaques is a 39-43 residue beta-amyloid peptide (Aß) (1). There is ample evidence to support the role of Aß and the cerebral plaques it forms in the pathogenesis of Alzheimer's disease (2).

Soluble Aß exists free in the blood and cerebrospinal fluid and eventually deposits as "insoluble" aggregates to produce cerebral plaques. Small alterations in the levels of soluble Aß throughout the body may determine whether a person remains healthy or develops Alzheimer's disease. We therefore propose to develop catalytic antibodies that will specifically recognize and efficiently destroy Aß. These unique reagents could then be used therapeutically to reduce Aß levels in both the blood and the brain. The specific aims of this research are to:

1- Construct Transition State Aß Antigens
2- Produce Catalytic Anti-Aß Monoclonal Antibodies
3-Test Catalytic Anti-Aß for Disrupting Plaque Development in Mice

More than 20 years ago, Raso and Stollar published the first study expressly aimed at inducing antibodies possessing catalytic activity (3). A transition state enzyme inhibitor was designed, synthesized and used elicit complementary antibody combining sites that would mimic an enzyme active site. With the emergence of monoclonal antibody techniques, the field of catalytic antibodies has exploded, largely due to recent efforts from the laboratories of Lerner, Benkovic and Schultz.

We are developing new transition state analogs of Aß for the purpose of eliciting catalytic antibodies that will specifically cleave Aß into inactive fragments. Studies indicate that the deposit of full-length $Aß_{1-43}$ in senile plaques (4) is central to Alzheimer's disease pathogenesis and shortened versions of Aß may be less of a problem in the absence of $Aß_{1-43}$. Cleavage of $Aß_{1-43}$ by a catalytic antibody should yield harmless non-amyloidogenic pieces. Each antibody would continuously function to permanently inactivate many target molecules. Thus, attenuating or abolishing the plaque forming capability of Aß and lowering its level in the brain should curtail the progression of Alzheimer's disease.

The effect of these catalytic antibodies on Aß plaques will be studied in a transgenic mouse model of Alzheimer's disease (5). The catalytic antibodies can be administered systemically in order to greatly reduce circulating levels of Aß and thus to subsequently lower the intercommunicating pool of Aß in the brain. Alternatively, the anti-Aß catalytic antibodies can be introduced directly into the brain so that they come into immediate contact with cerebral Aß and Aß plaques. Delivery into the brain can be accomplished either by intracerebral infusion (6) or via transcytosis across the blood-brain barrier using a vectorized bispecific antibody construct (7). Aß levels will be measured by ELISA and amyloid plaques will be evaluated in brain sections.

The novel catalytic antibodies produced should help to combat Alzheimer's disease by providing a safe and effective means to destroy Aß in the body.

E. WORK PLAN *(Instructions: ctrl+alt E)*

Transition State Aß Antigens The amino acid sequence of beta-amyloid (Aß) is;

DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIAT
1 10 20 30 40

Predicting precisely which cleavage sites on this 43-residue Aß peptide will be best suited for catalytic antibody-mediated therapy is difficult. Therefore key regions of the Aß 43-mer were chosen as positions for modification with transition state residues. Those sites will serve as antigenic epitopes and subsequently as targets for cleavage of the unmodified Aß molecule by the induced catalytic antibodies.

We chose to synthesize long Aß transition state peptides because of two important considerations. An extended sequence is required to ensure that the predetermined cleavage specificity of the resulting catalytic antibody will exclusively recognize the Aß peptide. However, examination of naturally occurring proteases suggests that chain length can also play an important role in the catalytic mechanism. X-ray crystallography studies provide structural evidence for multiple enzyme-peptide interactions along elongated groove active sites. There are also many examples (8) where extension of the peptide substrate increases the k_{cat}/K_m 2000-4000-fold. By analogy we designed long transition state peptide analogs to induce complementary elongated antibody combining sites which mimic proteolyic enzyme active site clefts. Multiple contact areas between the antibody and peptide in an extended groove may be more effective for inducing strain in the peptide bond, which should promote its hydrolytic cleavage.

A terminal Cys residue was added to the transition state analogs to provide a sulfhydryl linkage group for coupling the peptides to antigenic, maleimide-activated carrier proteins such as Keyhole Limpet Hemocyanin (KLH). Peptides were purified by HPLC and mass spectral and amino acid analysis verified their composition.

These new transition state Aß antigens have been used to elicit a unique panal of monoclonal antibodies. Select anti-Aß catalytic antibodies will be identified, characterized and used for studies in transgenic (Tg) mice. To facilitate transport across the blood-brain barrier they will also be vectorized by coupling to an anti-transferrin receptor antibody (anti-TfR). The Tg mouse model will help to determine empirically which of these unique immuno-reagents are both therapeutically effective and pharmacologically safe.

A phosphonamidate transition state analog encompassing the carboxy-terminal region of Aß has been synthesized. ↓

N-acetyl-Cys-Met-Val-Gly-Gly--**CO-NH**--Val-Val-Ile-Ala-amide

35 40

N-acetyl-Cys-Met-Val-Gly-Gly-**PO_2^--NH**-Val-Val-Ile-Ala-amide

Replacement of the proposed scissile peptide linkage between Gly_{38} and Val_{39} (↓) with a phosphonamidate moiety (**-PO_2^--NH-**) is designed to elicit catalytic antibodies that will hydrolytically cleave Aß at this site. The N-acetyl-Cys residue was placed at the position of Leu_{34} to provide a suitable linkage group for coupling this peptide to an antigenic carrier protein. The structures in Fig. 1 represent the putative transition state for peptide hydrolysis by zinc peptidases and the phosphonate and phosphonamidate mimics. Tetrahedral transition state intermediates are formed in all proteolytic enzymes, the serine-, cysteine-, aspartic- and metallo-peptidases.

E. WORK PLAN—PAGE 2

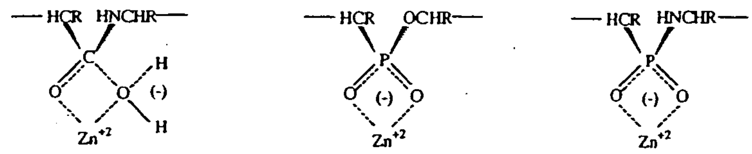

Fig. 1 Transition-state Phosphonate Phosphonamidate

The design strategy and methods for synthesizing phosphonamidate and phosphonate based transition state peptides are straightfoward (9). The N-terminal portion of the peptide (N-acetyl-Cys-Met-Val-Gly) was made using standard automated Fmoc chemistry. Its amino terminus was capped with acetic anhydride while it was on the resin. After cleavage it was treated with pyridine disulfide to protect its sulfhydryl group. An acid chloride of Cbz-glycine phosphonate monomethyl ester, was coupled with Val-Val-Ile-Ala-amide which was synthesized by Fmoc chemistry. The Cbz-Gly-**PO_2^--NH**-Val-Val-Ile-Ala-amide has a phosphonamidate (methyl ester) bond between the Gly and Val residues. Next, the Cbz blocking group was removed using hydrogen so that the protected N-acetyl-Cys-Met-Val-Gly peptide could be added to the amino terminal end by HBTU-activated peptide linkage. The peptide was then deblocked and coupled to maleimide-activated KLH.

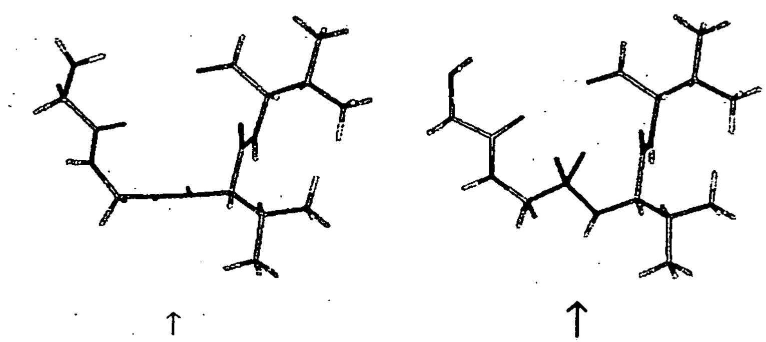

Fig. 2 Gly-Gly-**CO-NH**-Val-Val Gly-Gly-**PO_2^--NH**-Val-Val

A structural comparison was made between the native Aß peptide and the transition state phosphonamidate Aß peptide using a graphics workstation. The peptide link **-CO-NH-** (↑) between Gly_{38} and Val_{39} was replaced with a phosphonamidate bond **-PO_2^--NH-** (↑) and an energy minimization was applied. Fig. 2 clearly illustrates the planar peptide link **-CO-NH-** (↑) of native Aß (left) versus the tetrahedral phosphonamidate bond **-PO_2^--NH-** (↑) in the transition state peptide (right).

An antibody combining site complementary to the tetrahedral transition state analog on the right of Fig. 2, will force the normally planar bond of the Aß substrate peptide on the left into a transition state-like conformation. Such bond distortion could catalyze the hydrolytic cleavage of the Aß peptide at the Gly_{38}-Val_{39} linkage.

A series of statine (**Sta**) transition state analogs encompassing the carboxyl-terminal region of Aß (Cys-Met-Val-Gly-Gly-Val/**Sta**-Val/**Sta**-Ile/**Sta**-Ala-Thr) was synthesized in this laboratory.

Met-Val-Gly-Gly-Val↓Val↓Ile↓Ala-Thr-CO_2H
35 40

E. WORK PLAN—PAGE 3

Replacement of the proposed scissile peptide linkage between Val_{39} and Val_{40} (↓), Val_{40} and Ile_{41} (↓) and Ile_{41} and Ala_{42} (↓) with a tetrahedryl "statyl" moiety (**-CHOH-CH$_2$-CO-NH-**) is designed to elicit catalytic antibodies that will hydrolytically cleave Aß at one of these sites. A Cys residue was placed at the position of Leu_{34} to provide a suitable linkage group for coupling this peptide to a maleimide-activated carrier protein. The statine transition state peptides were entirely synthesized using standard automated Fmoc chemisty. This was facilitated by commercially available Fmoc-statine, [N-Fmoc-(3S,4S)-4-amino-3-hydroxy-6-methyl heptanoic acid].

A series of phenylalanine statine (**PhSta**) transition state analogs encompassing the central region of Aß (Cys-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe/**PhSta**-Phe/**PhSta**-Ala-Glu-Asp-Val-Gly-amide) was synthesized in this laboratory.

Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe↓Phe↓Ala-Glu-Asp-Val-Gly-amide
10 15 20 25

Replacement of the proposed scissile peptide linkage between Phe_{19} and Phe_{20} (↓) and Phe_{20} and Ala_{21}(↓) with a statyl moiety (**-CHOH-CH$_2$-CO-NH-**) is designed to elicit catalytic antibodies that will hydrolytically cleave Aß at these sites. A Cys residue was placed at the position of Gly_9 to provide a sulfhydryl linkage group for coupling the peptide to antigenic, maleimide-activated KLH. The phenylalanine statine transition state peptides were synthesized using standard automated Fmoc chemisty. This was feasible due to the recent availability of Fmoc-"phenylalanine statine," [N-Fmoc-(3S,4S)-4-amino-3-hydroxy-5-phenylpentanoic acid].

An antibody combining site that is complementary to the elongated tetrahedral transition state analog will force the normally planar peptide bond of the Aß substrate into a transition state-like conformation. Such distortion should catalyze the hydrolytic cleavage of Aß at the Phe_{19}-Phe_{20} bond.

Anti-Aß Monoclonal Antibodies Standard protocols were used to immunize mice with the KLH-linked transition state Aß. We performed a hybridoma fusion using the spleen of a mouse immunized with the phenylalanine statine transition state Aß-KLH antigen. Monoclonal antibodies from the hybridoma supernatants were screened using ELISA to assess their binding to both the normal $Aß_{1-43}$ peptide and to the phenylalanine statine transition state Aß peptide. Two patterns were found (Fig. 3).

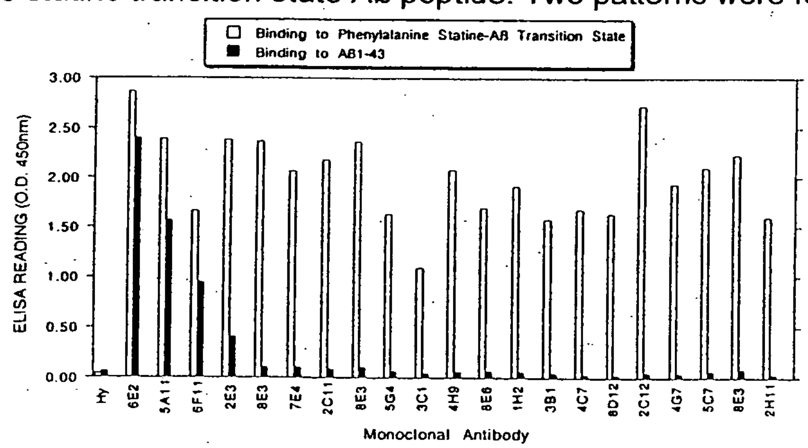

Fig. 3 ELISA Comparing Antibody Binding to Transition State Versus Native Aß

E. WORK PLAN—PAGE 4

One group of antibodies (at the left of Fig. 3) bound to the immunizing transition state peptide and cross-reacted strongly with the native Aß$_{1\text{-}43}$ peptide when each was adsorbed directly onto the ELISA plate. A second group (at the right) showed a high binding preference for the phenylalanine statine transition state Aß peptide and reacted minimally with native Aß$_{1\text{-}43}$. The anti-peptide specificity of the antibodies was ensured since they exclusively bound to the carrier-free Aß peptides.

These findings indicate that several of the new anti-Aß transition state antibodies are unique. They can bind to both the phenylalanine statine- and normal-Aß peptides. Their selective recognition of the transition state and weaker cross-reaction with native Aß$_{1\text{-}43}$ however implies that this binding interaction is very different from that shown by conventional anti-native Aß antibodies. It suggests further that these new antibodies may be able to force the native Aß peptide into a conformation resembling the transition state for hydrolytic cleavage.

Another distinct hybridoma fusion was performed using the spleen of a mouse immunized with a KLH conjugate of the statine (**Sta**) transition state analogs encompassing the carboxyl-terminal region of Aß (Cys-Met-Val-Gly-Gly-Val/**Sta**-Val/**Sta**-Ile/**Sta**-Ala-Thr). ELISA was used to demonstrate antibody binding to both the normal Aß$_{1\text{-}43}$ peptide and to the statine transition state Aß peptide (not shown). The antibodies bound to the C-terminal locus on these carrier-free Aß peptides adsorbed directly to the microtitre plate, confirming their anti-peptide specificity. Most of the antibodies preferentially recognized the statine Aß transition state but cross-reacted with native Aß$_{1\text{-}43}$. This suggests that these new antibodies may be able to force the native Aß peptide into a conformation resembling the transition state for hydrolytic cleavage of its C-terminal amino acids. Used therapeutically, such catalytic antibodies would, in effect, convert the less abundant but more noxious Aß$_{1\text{-}43}$ species into potentially less harmful shorter peptides, like Aß$_{1\text{-}40}$ or Aß$_{1\text{-}39}$.

A solid phase ^{125}I-labeled Aß assay was developed to screen anti-transition state antibody hybridoma supernatants for specific proteolytic activity. A peptide encompassing amino acids 14-25 of Aß was synthesized with a Cys and Tyr added at either end. This was radiolabeled with ^{125}I and the iodinated peptide was then separated from unlabeled material by HPLC to give essentially quantitative specific activity. The highly radioactive Aß peptide was coupled to a thiol-reactive, iodoacetyl-Sepharose gel to form an irreversible linkage. Catalytic antibodies should promote the progressive release of soluble ^{125}I-peptide from the solid phase matrix. The proposed assay was verified by the ability of several different proteases to rapidly hydrolyze this Sepharose-linked ^{125}I-Aß substrate.

Selected antibodies were screened for catalytic activity using release of radioactivity from ^{125}I-Aß-Sepharose (Fig. 4). The results obtained at pH 7, 25°C indicate that the antibody-containing media of several clones released ^{125}I-peptide at a greater rate than other clones from this fusion or the PBS and Hy medium controls (Fig. 4). Large amounts of these antibodies will now be obtained, purified and tested at higher concentrations to achieve much faster rates of cleavage and to verify that the antibodies are acting in catalytic mode by conventional enzyme kinetics.

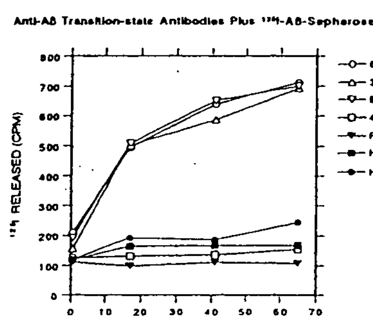

Fig. 4

E. WORK PLAN—PAGE 5

Treatment of Transgenic "Alzheimer's mice" The age-dependent appearance of neurological symptoms in the Tg2576 transgenic (Tg) mice (breeders provided by Dr. K. Hsiao) (5) allows for experimental intervention. Mice < 9-months old have low levels of Aß in the brain, no Aß plaques and good memory. Mice > 9-months old have high brain levels of Aß, many plaques (Fig. 5), and do poorly in memory tests (5).

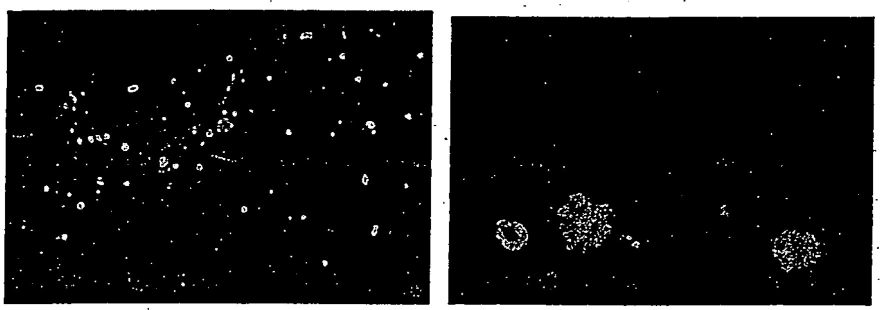

Fig. 5 Low and Hi Power Thioflavin Stained Brain Plaques from One of Our Tg Mice

Our strategy is to use anti-Aß catalytic antibodies to treat both young Tg mice which have not yet developed Aß plaques and old Tg mice which have already developed high levels of cerebral Aß and brain plaques. The first case tests if the catalytic antibodies can delay or prevent the formation of plaques while the second asks if the antibodies can diminish or eradicate preestablished plaques (Fig. 5).

Anti-Aß catalytic antibodies will be used either systemically to lower circulating Aß levels, infused directly into the brain (6) or delivered into the brain as a vectorized antibody that can cross the blood-brain barrier (7). Thus, a bispecific anti-Aß/ anti-mouse transferrin receptor antibody was produced (10). Dual specificity was verified by its attachment to receptor bearing cells and simultaneous binding of ^{125}I-Aß$_{1-40}$.

Table I Bispecific Antibody-Mediated Binding of ^{125}I-Aß to Transferrin Receptor-Positive Cells

Pretreatment of Cells	^{125}I-Aß Bound (cpm)
None	2,367
+ anti-Aß/anti-transferrin receptor	11,476

Different groups of transgenic mice will be injected i.p. with 1mg of catalytic antibodies, vectorized antibodies or appropriate non-specific control antibodies, biweekly for 2 months. Intracerebral antibody infusions (1µl, 50mg/ml) will be via a brain cannula (6), stereotaxically placed by Taconic Technical Services. Serum or brain Aß concentrations will be measured by ELISA (5) and Aß plaques in brain sections will be evaluated by immunocytochemical and thioflavin S staining (Fig. 5).

1. D. J. Selkoe, et al., *J. of Neurochemistry* **46**, 1820-1834 (1986)
2. C. Haass and D. J. Selkoe, Cell **75**, 1039-1042 (1993)
3. V. Raso and B.D. Stollar, *Biochemistry* **14**, 584-591 (1975)
4. S.A. Gravina, et al., 1995. *J. of Biol. Chem.* **270**, 7013-7016 (1995)
5. K. Hsiao, et al., *Science* **274**, 99-102 (1996)
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8. G. P. Sachdev and J. S. Fruton, *Biochemistry* **9**, 4465-4470 (1975)
9. P.A. Bartlett and C.K. Marlow, *Biochemistry* **26**, 8553-8561 (1987)
10. V. Raso, et al., *J. Biol. Chem.* **272**, 27618-27628 (1997)

F. AVAILABLE RESOURCES & BUDGET JUSTIFICATION

(Instructions: ctrl+alt F)

Resources: The PI occupies 1100 sq. ft. of laboratory and office space on the 2nd floor of the Boston Biomedical Research Institute and has access to 900 sq. ft. of common space. A complete small animal facility, including a room dedicated to breeding and handling transgenic mice, is located in the basement of the Institute.

BBRI has a Microvax and we are connected to the Internet. Several desktop computers are located in the lab and office for image processing, data analysis, literature searches, sequence analysis, etc. The Silicon Graphics Iris Indigo XS24 graphics workstation at BBRI is available to this project for molecular modeling.

BBRI has a well equipped machine shop, an in-house library and access to the Treadwell library at the Massachusetts General Hospital.

The BBRI has a PerSeptive Biosystems Voyager RP MALDI-TOF mass spectrometer. An in-house fluorescence flow cytometry facility is available. We have a molecular imager for acquiring and digitizing radioisotopic brain images.

An in-house Morphology Unit provides Histology, Electron Microscopy and Confocal Microscopy Services. BBRI also has an X-ray crystallography facility.

We have access to all of the necessary equipment to perform the intracerebral infusion experiments described in the proposal.

Within the laboratory of the PI are: a scintillation counter, pH meter, semi-micro balance, top loading balance, Zeiss inverted tissue culture microscope, CO2 incubator, fraction collectors, UV monitor, LKB spectrophotometer, microscope, clinical centrifuge, 6 ft. laminar flow hood, 1 liquid N2 tank, flash evaporator, horizontal DNA/RNA gel electrophoresis apparatus, 2 microfuges, PAGE apparatus, a western blot apparatus, HPLC, and a video fluorescence microscope set-up.

Shared equipment at BBRI includes: a gamma counter, 3 scintillation counters, 4 ultracentrifuges, a peptide sequencer, 2 autoclaves, 3 cold rooms, 3 spectrophotometers, a DNA synthesizer, a peptide synthesizer, 3 PCR machines, 4 HPLC units, an electron microscope, a circular dichroism spectrophotometer, a florescence lifetime apparatus, a DU 650 spectrophotometer, a phosphoimager apparatus, and an ELISA plate reader.

Personnel: The principal investigator will devote 20% time/effort to supervise the overall project and the scientific endeavors of the research technician, Christine Kearney (50% time/effort). The P.I. and research technician will take responsibility for the production of anti-beta-amyloid catalytic antibodies and their characterization. They will then evaluate these antibodies in a transgenic mouse model of Alzheimer's disease. The P.I. and research technician will:

- design, synthesize and purify several transition state Aß peptides
- carry out mass spectral and amino acid analyses
- link transition state Aß peptides to antigenic carriers
- immunize mice with the transition state Aß antigens
- screen immunized mice for the production of anti-Aß antibodies
- perform hybridoma fusions using the spleenocytes from immunized mice
- use ELISA to screen hybridoma clones for anti-Aß transition state antibodies

F. AVAILABLE RESOURCES & BUDGET JUSTIFICATION- Page 2

- develop catalytic antibody screening assays to detect cleavage of the Aß peptide
- collect ascites from mice for monoclonal antibody isolation
- purifiy antibodies and fully define their specificity and catalytic activity
- analyze the peptide cleavage products produced by the catalytic antibodies
- couple purified anti-Aß to purified anti-transferrin receptor antibodies
- verify the dual specificity of the vectorized bispecific antibodies
- perform genetic analyses on the transgenic "Alzheimer's mice" (Tg mice)
- treat Tg mice i.v. with catalytic antibodies or bispecific antibodies
- carry out catalytic anti-Aß infusion studies in Tg mice fitted with cerebral cannulas
- use ELISA to measure Aß levels in serum and brain extracts
- evaluate plaques in brain sections by immunocytochemical and thioflavin S staining
- test catalytic anti-Aß antibodies for their ability to displace Aß equilibria in Tg mice
- examine Tg mice for the effects of catalytic antibodies on the formation of plaques
- test catalytic anti-Aß antibodies for delaying or reversing Aß plaque formation
- examine Tg mice for adverse effects due to treatment with catalytic anti-Aß
- run ELISA and radioimmunoassays
- order and maintain laboratory reagents and supplies

Supplies: The experimental studies planned will require ample supplies for the peptide/organic synthesis of several different transition state beta-amyloid antigens. Chemistry supplies and reagents are also needed for the purification and analysis of the peptides. Cell culture materials are required for the hybridoma fusions and for maintenance of the hybridoma clones. Chromatography materials will be used for the isolation of the new peptides, the antigen conjugates and the monoclonal antibodies. Immunochemicals are needed to perform the ELISA and radioimmunoassay protocols for monitoring antibody production in the transgenic mice. The purchase of animals for this project is also included in the supply costs.

Other Expenses: The ongoing breeding of transgenic mice and long term housing of the Tg mouse colony is reflected in the animal costs. Surgical services for stereotaxic cannula placement in the transgenic mice cost $37/mouse. Publication charges are also requested.

Duration of Support: Three years of support have been requested. I believe that the productivity and pioneering discoveries of my laboratory has proven our long-term commitment to the study of immunology and to the development of new strategies for realizing its clinical usefulness. The proposed research is fairly straightforward but substantial time and effort will be required to achieve our goals. The duration requested will allow us to follow up on our preliminary findings and continue to gain new and important scientific insights. Moreover, the research has been designed so that its fundamental findings may have a direct application to Alzheimer's disease. Fringe benefits are calculated at 29% for professional and non-professional personnel. Indirect costs are calculated at 92% based on salary and wage.

G. COST ESTIMATE *(Instructions: ctrl+alt G)*

Categories	YEAR 1	YEAR 2	YEAR 3
Personnel			
Salaries: List here each member of the team plus % of time (FTE) allocated to the project			
Victor Raso (20%)	20,080	20,080	20,080
Chris Kearney (50%)	13,060	13,060	13,060
Benefits: List here each member of the team plus % of time (FTE) allocated to the project			
Victor Raso (20%)	5,823	5,823	5,823
Chris Kearney (50%)	3,787	3,787	3,787
Lab and Research (e.g. supplies including animals)	7,800	7,800	7,800
Consultants	0	0	0
Publication Costs	300	300	300
Travel (≤$1000)	0	0	0
Subcontracts	0	0	0
Other	3,695	3,695	3,695
Total Direct Costs	54,545	54,545	54,545
Indirect Costs *Maximum of 10% of direct costs*	5,455	5,455	5,455
Total Direct & Indirect Costs	**60,000**	**60,000**	**60,000**
Cost Sharing			

H. APPLICANT INFORMATION: SCIENTIFIC, TECHNICAL AND ACADEMIC QUALIFICATIONS *(Instructions: ctrl+alt H)*

Principal Investigator: Victor A. Raso, Ph.D.

Professional Experience:

1973-1974	Associate in Pharmacology, Harvard Medical School
1973-1981	Research Associate, Division of Biochemical Pharmacology, Dana-Farber Cancer Institute
1981-1988	Assistant Professor of Pathology, Dana-Farber Cancer Institute, Harvard Medical School
1988-1989	Principal Scientist, Boston Biomedical Research Institute
1989-	Senior Scientist, BBRI, Dept. of Cell & Molecular Biology

Publications (Partial):

1. Raso, V and Stollar, BD. Antibodies specific for conformationally distinct coenzyme-substrate transition state analogues. A fluorescence, N.M.R., circular dichroism and antibody study of N-(5-phosphopyridoxyl)-3'-amino-L-tyrosine. J. Amer. Chem. Soc. 1973; 95:1621.
2. Raso V and Stollar BD. The antibody-enzyme analogy. Characterization of antibodies to phosphopyridoxyltyrosine derivatives. Biochem. 1975; 14: 584-91.
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7. Raso V. Antibody mediated delivery of toxin molecules to antigen bearing target cells. In: Moller, G, Ed. Immunological Reviews; 1982: 93-117.
8. Raso V. and McGrath J. Diphtheria toxin cures athymic mice of human malignant mesothelioma. J. Natl. Cancer Inst. 1989; 81, 622-627.
9. Recht, L., Torres, C. O., Smith, T. W., Raso, V. A., and Griffin, T. W. (1990) Transferrin receptor in normal and neoplastic brain tissue: Implications for brain-tumor immunotherapy. *J Neurosurg* **72,** 941-945.
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11. Recht, L., Raso, V. Davis, R. and Salmonsen, R. Immunotoxin sensitivity of CHO cells expressing human transferrin receptors with differing internalization rates. Cancer Immunol. Immunotherpy. 1996; 42: 357-361
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14. Raso, V., Brown, M. and McGrath, J. Intracellular targeting with low pH triggered bispecific antibodies. J. Biol. Chem. 1997; 272: 27623-27628.

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Application Number *(for office use only)*:
Project Title:

I. APPLICANT INFORMATION: LIST OF CONFLICTS

(Instructions: ctrl+alt I)

REVIEWER CONFLICTS: *(Use Font Size 8)*				
Name	Address	Phone	FAX	E-Mail

J. APPLICANT INFORMATION: TECHNICAL REVIEWERS

(Instructions: ctrl+alt J)

REVIEWERS: *Please provide 15 individual contacts (Use Font Size 8)*				
Name	Address	Phone	FAX	E-Mail
Dr. R. E. Tanzi	Dept. Neurol., Massachusetts Gen. Hosp., Charlestown, MA 02129	(617) 726-6845	(617) 726-5736	tanzi@helix.mgh.harvard.edu
Dr. Thomas Griffin	Oncology/Immunology, Hoffman-LaRoche, Inc., 340 Kingsland St, Nutley, NJ 07110-1199	(973) 562-3460	(973) 235-4044	tom.griffin@roche.com
Dr. B. D. Stollar	Dept. Biochem.,Tufts U Sch. Med., 136 Harrison Ave., Boston, MA 02111-1800	(617) 636-6868	(617) 636-6409	dstollar@opal.tufts.edu
Dr. John E. Maggio	BCMP Department Harvard Med Sch 240 Longwood Ave, Boston MA 02146	(617) 432-0757	(617) 432-3833	maggio@bcmp.med.hrvard.edu
Dr. Karen Hsiao	Dept. Neurol., UMHC, Box 295, 420 Delaware St., U Minnesota, Minneapolis, MN 55455	(612) 625-9900	(612) 625-7950	hsiao005@maroon.tc.umn.edu
Dr. Lawrence Recht	Dept. Neurol., U Massachusetts Med Center, 55 Lake Ave, Worcester, MA 01655	(508) 856-4147	(508) 856-6778	lawrence.recht@banyon.ummed.edu
Dr. Paul M. Knopf	Dept. Mol. Microbiology and Immunology,Brown Univers, Box G-B413, Providence, RI 02912	(401) 863-1218	(401) 863-1971	Paul_Knopf@Brown.edu
Dr. Abba J. Kastin	Dept. Med. Tulane School of Med 1601 Perdido St., New Orleans, LA 70146	(504) 568-0811 ext. 5884	(504) 522-8559	
Dr. Peter G. Schultz	Dept. Chem. U-Cal-Berkeley, 003068, 824 Latimer Hall, Berkeley, CA 94720	(510) 642-9277	(510) 643-6890	Pgschultz@LBL.gov

Keyword List

(Type of) Glutamate Receptor
Acetylcholine Anatomy /
Activities of Daily Living
Adeenergic System
Adult Day Care
Aluminum
Amyloid Diseases
Amyloid Precursor Protein
Anatomy
Animal Behavior
Animal models
Animal Models-Trangenics
Antidepressants
Antisense Oligonucleotides
Anxiety
Apolipoprotein
Apoptosis
Autonomic mechanisms
Axonal Transport
Behavioral Effects
Behavioral Management
Behavioral Neurology
Behavioral Pharmacology
Beta-amyloid
Biochemistry
Biological Assays
Blood Brain Barrier
Calcium Imaging
Caregiving Issues
Cell Biology
Cell Cultures
Cell Death
Cellular Physiology
Cerebral Metabolism
Cerebrospinal Fluid
Cholinergic System
Circadian rhythms
Clinical Assessment
Clinical Care
Clinical Diagnosis
Clinical Pharmacology
Clinical Trials
Cloning
Cognition

Cognitive Neuroscience
Cognitive Psychology
Cognitive Therapy
Communication/Language
Cost Benefit Analysis
Cytoskeleton
Dentistry
Depression
Developmental Neurobiology
Disabilities
DNA Sequencing
Down Syndrome
Drosophila Neurobiology
Drug Delivery
Drug Design
Drug Metabolism
Elder Abuse
Electroencephalography
Electron Microscopy
Electrophysiology
Endocrinology
Enzyme linked immunosorbent
Enzymology
Epidemiology
Estrogen
Ethical Issues
Event related potential
Excitotoxins
Familial Alzheimer's Disease
Forebrain
Free Radicals
GABA Receptors
Gangliosides
Gene Cloning
Gene Expression
Gene Mapping
Gene Transfer
Genetic Linkage
Genetics
Gerontology
Glutamate
Glutamate Receptors
Glycoproteins
Growth Factors

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Health Care Financing
Health Facilities & Services
Health Financing
Hemostasis
Herpes Simplex Virus
Hippocampus
Histochemistry
Histopathology
Human Genetics
Immunoassay
Immunocytochemistry
Immunohistochemistry
Immunology
In Situ Hybridization
In Vitro Methods
In Vivo Microdialysis
Instrument Development
Ion Channels
Language
Legal Issues
Lewy Bodies
Linkage Analysis
Lipid Metabolism
Long Term Potentiation
Longitudinal Study
Magnetic Reson.
Magnetic Resonance Imaging
magnetoencephalography
Mass Spectroscopy
Medicinal Chemistry
Medicine
Membrane Biochemistry
Memory
Messenger RNA
Microtubules
Microvessels
Molecular Biology
Molecular Genetics
Morphology
Movement Disorders
MR spectroscopic imaging
Multicultural Issues
Muscarinic Receptors

Nerve Growth Factor
Neural Degeneration
Neural Development
Neural System
Neural System-Olfactory
Neural System-Visual
Neural Transplantation
Neural-Viral Interactions
Neuroanatomy
Neurobiology
Neurochemistry
Neuroendocrinology
Neurofibrillary Tangles
Neurofilaments
Neuroimmunology
Neurology
Neuromorphology
Neuronal Cell Death
Neuroopthalmology
Neuropathology
Neuropharmacology
Neurophysiology
Neuropsychological Testing
Neuropsychology
Neurosurgery
Neurotoxicology
Neurotrophic Factors
Nicotine
Non-Cognitive Behavior
Normal Aging
Northern Blot Analysis
Nuclear Magnetic Resonance
Nursing
Nursing Home Research
Nutrition
Oral Health
Otolaryngology
Oxidative Injury
Paired Helical Filaments
Parkinson's Disease
Patch Clamp
Pathology
Patient Care

Pharmacokinetics
Pharmacology
Physical Chemistry
Physiological Psychology
Physiology
Plaques
Policy Analysis
Polymerase chain reaction
Positron Emission
Presymptomatic Testing
Primates
Prion Proteins
Program Evaluation
Proteases
Psychiatry
Psychology
Psychopharmacology
Qualitative Methods
Quantitative Autoradiography
Quantitative RNA/DNA
Radiology/Neuroimaging
Radiopharmaceuticals
Receptors
Regeneration (Sprouting)
Respite Care
Risk Factors
RNA Analysis
Second Messengers
Senescence
Signal Transduction
Single Unit Recording
Sleep
Social Work
Sociology
SPECT-Single Photon Emis,
Staffing/Training Issues
Staging of Dementia
Statistical Procedures
Surgery
Surveys
Tau Protein
Tissue Cultures
Trace Elements

Transgenic Mice
Trisomy
Trophic Factors
Vascular Disease
Videotaping
Virology
Vision
Visuospatial Attention
Voltage Clamp

faxed 9/3/98

BOSTON BIOMEDICAL RESEARCH INSTITUTE

Victor A. Raso, Ph.D.

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Area code 617 • 742-2010
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Sept. 1, 1998

Mr. Nico Stanculescu
Research Grants Program of the
Alzheimer's Association

Dear Mr. Stanculescu,

I am sending you this letter-of-intent in advance of preparing an application for an Investigator Initiated Research Grant. The proposed research will be carried out at the Boston Biomedical Research Institute where I hold a senior scientist position. This project is aimed at the pre-clinical development of an immunological approach for the treatment and/or the prevention of Alzheimer's disease. It is titled "Vaccine to Modulate Systemic ß-Amyloid Levels" and encompasses three major specific aims.

- To Produce ß-Amyloid Peptide Vaccines
- To Immunize Transgenic Mice with the ß-Amyloid Peptide Vaccines
- To Test for the Disruption or Prevention of Cerebral Plaque Development in Vaccinated Mice

Thank you for your kind consideration.

Very truly yours,

Vic Raso

Victor Raso, Ph.D.

B000432

KRS: *APPLICATION FORM FOR INDIVIDUAL RESEARCH PROJECT©*
Application Number *(for office use only)*:
Project Title:

A. APPLICANT MANAGEMENT INFORMATION *(Instructions: ctrl+alt A)*

Principal Investigator

Name	Credentials	Social Security No. (if non-US national Passport No.)	
Victor A. Raso	Ph.D.	050-36-7225	
Address	**E-mail Address**	**Phone**	**FAX**
Boston Biomedical Research Institute 20 Staniford St. Boston, MA 02114	Raso@bbri.harvard.edu	617, 912-0316	617, 912-0308

Scientific Staff: Collaborators and Consultants

Name (Affiliation)	SS# (Passport #)	Name (Affiliation)	SS# (Passport #)

Fiscal Responsibility (business official who will have fiscal responsibility for this application)

Name	Credentials	Social Security No. (if non-US national Passport No.)	
Thomas J. McQuaid	MBA, MST, CPA	024-44-8367	
Address	**E-mail Address**	**Phone**	**FAX**
Boston Biomedical Research Institute 20 Staniford St. Boston, MA 02114	McQuaid@bbri.harvard.ed u	617, 912-0301	617, 912-0335

Institution Approval Requirements: Please Mark the Appropriate Box or Boxes with "XXX"

Human/Human Tissue Studies IRB APPROVAL WILL BE/HAS BEEN OBTAINED	Animal/Animal Tissue Studies IACUC APPROVAL WILL BE/HAS BEEN OBTAINED
	XXX

Grant Information

Dollar Amount Requested	Duration of Support	Start Date
$180,000	3 yrs.	04/01/99

Project Title

Vaccine to Modulate Beta-Amyloid Levels

Competition (IIRG=Investigator Initiated Research Grant, PRG=Pilot RG, HAT=Hatfield RG, ZEN=Zenith RG, PIO=Pioneer RG)

IIRG

B. PROJECT ABSTRACT *(Instructions: ctrl+alt B)*

The aim of this project conducted by Dr. Victor Raso at the Boston Biomedical Research Institute is to develop a vaccine for the treatment and/or prevention of Alzheimer's disease. It relies upon basic immunological principles and uses a vaccine approach to elicit peptide-specific antibodies designed to tightly bind systemic beta-amyloid. The amyloid peptide and the cerebral plaques that it forms are likely either the direct or indirect cause of Alzheimer's disease. Soluble amyloid exists free in the blood and cerebrospinal fluid while "insoluble" aggregates are deposited in the brain as amyloid plaques. The soluble and insoluble forms of beta-amyloid present within Alzheimer's patients appear to be in dynamic equilibrium. This equilibrium will be displaced away from the brain by deploying a vaccine designed to generate peptide-specific antibodies in a transgenic mouse model of Alzheimer's disease. Restricted to the peripheral circulation, these antibodies will trap the amyloid peptide in the blood and by doing so could gradually deplete intercommunicating peptide levels in the brain. Decreased concentrations of beta-amyloid in the brain should reduce the size and number of brain plaques or delay their appearance. These studies would establish whether or not there is a causal relationship between amyloid deposits and memory impairment in those transgenic mice. Moreover, this expressly designed vaccine and the anti-beta-amyloid antibodies it elicits could produce beneficial therapeutic effects by perturbing the critical amyloid balance in the mouse model. If successful this research using beta-amyloid vaccines would establish a scientific basis for the specific immunotherapy of Alzheimer's disease.

C. PROJECT KEYWORDS *(Instructions: ctrl+alt C)*

Project Keywords (Please choose from accompanying list where possible)	
Beta-amyloid	* Antibody
Immunology	* Immunize
Plaques	
Transgenic Mice	
* Vaccine	

D. PROBLEM STATEMENT *(Instructions: ctrl+alt D)*

Presently, there are few encouraging therapeutic prospects for the prevention or treatment of Alzheimer's disease. However, the equilibrium between soluble beta-amyloid peptide (Aß) and Aß aggregates in brain plaques suggest that an immunotherapeutic approach to the disease should be feasible. The objective of this project is to develop novel therapeutic vaccines which will elicit specific anti-Aß antibodies. By efficiently binding Aß in the blood stream these antibodies should perturb its systemic equilibrium, gradually deplete Aß levels in the brain and thus delay or reverse plaque formation. This new, innovative form of intervention might ultimately be used to prevent or significantly alter the course of Alzheimer's disease.

Immunotherapy is safe since no harmful drugs or foreign agents are required for treatment. Endogenous antibodies are highly specific and can be easily sustained for as long as needed, important features for combating Alzheimer's disease. This study will use synthetic Aß peptides to create vaccines designed to induce anti-Aß antibodies which will alter systemic Aß levels. We will then test these reagents for beneficial effects in a transgenic mouse model of Alzheimer's disease. Our research will establish whether there is potential for the clinical development of anti-Aß vaccines as uniquely precise and urgently needed therapeutics. The aims are to:

1-Produce Beta-Amyloid Peptide Vaccines
2-Immunize Transgenic Mice with the Aß Peptide Vaccines
3-Examine the Immunized Mice for Disrupted or Delayed Plaque Development

Diagnosis of Alzheimer's disease at autopsy relies on the presence of neuropathological brain lesions marked by a high density of senile plaques. The principal component of these plaques is a 39-43-residue beta-amyloid peptide (1). There is ample evidence to support the pathogenic role of Aß (2) which is present in the blood (3), cerebrospinal fluid, brain and peripheral tissues.

Important experiments suggest that a dynamic equilibrium may exist between soluble Aß and fibrillar Aß deposited as plaques in the brain. ^{125}I-Aß peptide bound specifically, reversibly and with high affinity to brain plaques suggesting that a steady-state equilibrium exists between Aß in plaques and Aß free in solution (4). Moreover, mild treatment with certain anti-Aß monoclonal antibodies can effectively dissolve preformed Aß aggregates by binding soluble Aß (5). The preceding observations speak to the central thesis of this proposal, namely that the onset of plaque formation, plaque size or the number of plaques might be influenced by reducing levels of free Aß in the brain and these may in turn be linked to Aß levels in the blood. Induced endogenous antibodies (Ab) in the circulation can not cross the blood-brain barrier. Thus, they will act as a sink that should gradually reduce Aß levels in the brain by pulling the equilibrium to the right (Fig. 1). Concentrations of antibody that are sustainable in animals (~1µM) would far exceed the levels of circulating Aß (~0.6nM) (3). More than 99% of Aß in the blood would be complexed (Ab:Aß) by an antibody with an association constant of 10^8 M^{-1}.

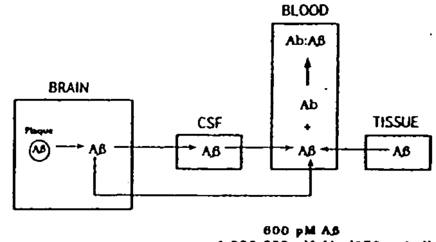

Fig. 1

E. WORK PLAN *(Instructions: ctrl+alt E)*

Beta-Amyloid Peptide Vaccines The amino acid sequence of beta-amyloid (Aß) is;

DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIAT
1 10 20 30 40

Predicting precisely which sites on this 43-residue Aß peptide will be ultimately best suited for antibody-mediated therapy is difficult. Therefore three key regions of the Aß 43-mer were chosen to generate epitope-specific vaccines. The segments selected as target sites were the amino-terminal, DAEFRHDSGYEVHHQKC; central, CYEVHHQKLVFFAEDVG and carboxyl-terminal, CMVGGVVIAT sequences. The C residue was placed at a terminal position to provide a sulfhydryl linkage group for coupling the peptides to an antigenic carrier protein.

We have synthesized the Aß 40-mer, the Aß 43-mer and the three small Aß peptides described above by standard automated Fmoc chemistry. Newly synthesized peptides were purified by HPLC and mass spectral and amino acid analysis verified their composition. The Aß 43-mer proved difficult to isolate so that a purified preparation was obtained from a commercial source. The peptide synthesizer, mass spectrometer and amino acid analyzer are all located at our BBRI facility so that the chemical synthesis of any additional Aß peptides can be readily accomplished if and when they are needed.

These shortened peptides will serve as antigenic epitopes to induce a highly specific antibody response. A more generalized immune response will be also be generated by immunizing either with a mixture of these small peptide antigens or by using the full-length 43 residue Aß peptide linked to Keyhole Limpet Hemocyanin (KLH) with glutaraldehyde. The transgenic mouse model will help to determine empirically which of these unique vaccines are both therapeutically effective and pharmacologically safe.

The small Aß peptides have been linked to the KLH carrier protein in order to render them antigenic. A Cys residue was strategically placed at the N- or C-terminal end of these Aß peptides to provide a suitable linkage group for coupling them via a thioether bond to maleimide activated carrier proteins. This linkage is stable and attaches the peptide in a defined orientation. Addition of ~25 peptides/KLH can be typically obtained by this conjugation method. The longer, full-length Aß peptides were linked to carrier proteins using a glutaraldehyde coupling procedure.

The outlined methods are an effective and expedient way of producing experimental vaccines for use in animals. For future use in patients however, modifications would have to be made in the Aß vaccines to render them more compatible for human use. Aß peptides, for example, can be genetically engineered into appropriate immunogenic carriers or into viral antigens and DNA vaccines could be designed to induce Aß immunization.

Vaccination of Transgenic "Alzheimer's mice" The age-dependent appearance of neurological symptoms in the Tg2576 transgenic mouse model offers some important opportunities for experimental intervention. Mice less then 9-months old had low levels of Aß$_{1\text{-}40}$ and Aß$_{1\text{-}43}$ in the brain, 48 pmol/gm and 13 pmol/gm, respectively, no amyloid plaques and good performance in the learning and memory tests (6). In contrast, mice over 9-months old had high brain levels of Aß$_{1\text{-}40}$ and Aß$_{1\text{-}43}$ (264 pmol/gm and 175 pmol/gm, respectively), many Aß plaques, and poor

E. WORK PLAN—PAGE 2

performance in learning and memory tests (6). Therefore the strategy for our studies is to use Aß vaccines to induce endogenous antibodies both in young, 2-3-month old transgenic mice which have not yet developed Aß plaques and in 1-year old transgenic mice which have already developed high levels of cerebral Aß and brain plaques. In first case we will be able to test if sustained high titre endogenous antibodies can delay or prevent the de novo formation of Aß plaques. The second situation will test whether sustained high titre endogenous antibodies can diminish or eradicate preestablished plaques.

Transgene-positive mice for breeding were generously provided by Dr. Hsiao (6). Our animal care technicians are well experienced in handling transgenic mice and have established a healthy colony of these animals. Dr. Hsiao recommended that we perform genotyping on the mice both before and after each experiment. Ear punch biopsy DNA has been prepared and PCR procedures have been carried out as reported (6) to identify mice bearing the transgene.

Different groups of transgenic mice will be injected i.p. with 50μg of either Aß peptide linked to KLH or unmodified KLH, in complete Freunds adjuvant. One month later, the mice will be given the Aß or control antigen i.p. in incomplete Freunds adjuvant. Thereafter, they will be periodically boosted i.v. with the appropriate antigen in PBS. Our experience has shown that this protocol will maintain adequate anti-Aß antibody levels over a long period of time. Some of these planned Aß and control immunizations of Tg2576 mice have already been initiated. To date the vaccinated Tg2576 mice have shown no ill effects due to the production of anti-Aß antibodies even though the older mice have preestablished cerebral Aß plaques.

Screening serum antibodies We will use both an ELISA and ^{125}I-Aß binding assay to monitor and characterize the antibody response elicited in the transgenic mice. This will allow us to identify animals displaying high titre anti-Aß antibodies with appropriate specificity for select epitopes on the peptide. Table I shows ELISA data for diluted serum from a non-immunized control mouse versus 1/100 and 1/1000 diluted serum from a mouse that was immunized with our central region Aß peptide-KLH vaccine. Carrier-free Aß peptide was adsorbed directly onto the microtitre plate so that anti-KLH antibodies in the serum would not be measured. The presence of bound anti-Aß peptide antibodies was revealed using a peroxidase-labeled anti-mouse IgG probe followed by the chromogenic substrate. Monoclonal antibodies have been raised against this central region Aß peptide and the hybridoma supernatants have also been successfully screened using this assay.

Table I ELISA for Binding to the Central Region Aß Peptide

Addition		Antibody Bound (O.D. 450nm)
Control Serum	1/100	0.527
Mouse 1 antiserum	1/100	3.465
Mouse 1 antiserum	1/1000	2.764

Another mouse was vaccinated with the carboxyl-terminal Aß peptide coupled to KLH. The spleen of this mouse was then used for a hybridoma fusion so that we could better characterize the specificity of its immune response (Fig. 2). Separate ELISAs were also used to screen the hybridoma clones generated and to demonstrate antibody binding to both the small carboxyl-terminal peptide $Aß_{35-43}$ and

E. WORK PLAN—PAGE 3

the full-length $A\beta_{1-43}$ peptide. The monoclonal antibodies bound to the carboxyl-terminal locus on each of these carrier-free Aß peptides adsorbed directly to the microtitre plate, confirming their anti-peptide specificity. We also tested the clones

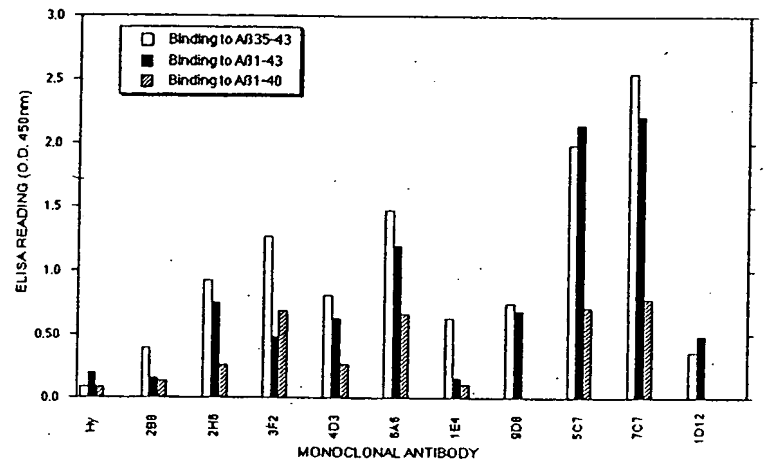

Fig. 2 ELISA Comparing Antibody Binding to $A\beta_{35-43}$ and $A\beta_{1-43}$ Versus $A\beta_{1-40}$

using $A\beta_{1-40}$ to identify antibodies which do not react with this shortened, 40 amino acid residue version of Aß and thus will specifically bind to the carboxyl-terminus of $A\beta_{1-43}$ (Fig. 2). Used therapeutically, this vaccine should elicit antibodies which will preferentially bind the less abundant but more noxious $A\beta_{1-43}$ species in the blood as opposed to the smaller and less detrimental $A\beta_{1-40}$.

It was important to demonstrate that the anti-Aß antibodies could also bind to the full-length Aß peptides in solution. Thus, we radiolabeled Aß with ^{125}I and then separated the iodinated peptide from unlabeled material by HPLC to give essentially quantitative specific activity (~2000 Ci/mmol) (4). The ^{125}I-Aß probe was incubated anti-Aß antiserum or monoclonal anti-Aß antibodies and then a polyethylene glycol separation method was used to detect the amount bound to antibody (Table II).

Aß in human plasma can bind to albumin or other carriers. We propose to use antibodies to sequester Aß in the blood and thereby displace the equilibrium away from the brain. It was therefore imperative to show that these antibodies can tightly bind Aß in the presence of a physiological level of human serum albumin (HSA). The data in Table II indicate that the ability of our purified 5A11 monoclonal anti-Aß antibody to bind ^{125}I-$A\beta_{1-40}$ was unaffected by HSA at 60mg/ml, even though this was a 500-fold molar excess over the antibody concentration. Therefore other

Table II ^{125}I-$A\beta_{1-40}$ Binding to Antibody in the Presence of Serum Albumin*

Addition	^{125}I-$A\beta_{1-40}$ Bound (cpm)	Specifically Bound (% of total added)
Control	8,560	-
+ 5A11 anti-Aß	64,589	79
Control + HSA*	3,102	-
+ 5A11 anti-Aß + HSA*	55,304	75

*Human Serum Albumin (HSA) at 60mg/ml (1mM); anti-Aß 5A11 at 2×10^{-6} M; Added 70,000 cpm of ^{125}I-$A\beta_{1-40}$

E. WORK PLAN—PAGE 4

binding proteins should not attenuate the ability of antibodies to tie-up Aß in blood.

Antibodies Affect the Distribution of ^{125}I-Aß in Mice Anti-Aß antibodies in the circulation cannot cross the blood-brain barrier to a significant extent and therefore should act as a sink that prevents ^{125}I-Aß$_{1-40}$ from reaching the brain. This retention effect was demonstrated by measuring the blood levels in mice 4 h after injecting them with equal amounts of ^{125}I-Aß$_{1-40}$ either alone or along with our 5A11 anti-Aß monoclonal antibody (Table III). The passage of ^{125}I-Aß$_{1-40}$ out of the peripheral circulation was greatly curtailed in animals that concomitantly received the specific anti-Aß antibody. That finding extends our *in vitro* results (Table II) by demonstrating that anti-Aß can also effectively bind Aß in an experimental animal. The fact that treatment with this injected antibody retained 10-times more ^{125}I-Aß$_{1-40}$ in the circulation lends credence to the premise that the equilibrium distribution of Aß in the body can be dramatically altered by its selective sequestration in the blood.

Table III Anti-Aß Impedes the Passage of ^{125}I-Aß$_{1-40}$ from the Circulation

Mouse Injected With:	^{125}I-Aß in Blood (cpm/gm)
^{125}I-Aß$_{1-40}$ alone	27,300
^{125}I-Aß$_{1-40}$ + 5A11 anti-Aß	278,900

Biodistribution of ^{125}I-Aß The ^{125}I-Aß probes will allow us to monitor the effect of immunization on the biodistribution of Aß in the transgenic mice. Passively administered anti-Aß antibodies sequestered ^{125}I-Aß injected into mice and retained it in the blood (Table III). Therefore this probe will be used in a similar manner to monitor the binding activity of circulating anti-Aß antibodies in the Aß-vaccinated and control transgenic mice. We expect that the anti-Aß antibodies in specifically immunized mice will bind the ^{125}I-Aß, hold it in the blood stream and prevent it from redistributing throughout the body. This would be reflected in the much higher levels of labeled peptide found in their blood compared to control mice (Table III).

Radioscintigraphy is being used to monitor ^{125}I-Aß entry and accumulation in the brain of live mice. Image data is obtained while the animal is chemically immobilized using ketamine/xylazine. This imaging technology might be very useful to determine if circulating anti-Aß antibodies will prevent i.v. administered ^{125}I-Aß from entering the brain. Importantly, digtital scintigraphy data can be easily quantified using standards and the integration functions provided in the analysis software. Sequential brain images from the same mouse can be acquired at 1, 6, 24 and 48 hours following i.v. administration of the ^{125}I- Aß probe.

Effect of Vaccination on Plaques and Memory Impairment The group of young mice which were immunized starting when they were 2 months old will be tested at the beginning of month 11 and on consecutive months thereafter. Mice from the experimental and control groups will undergo behavioral testing in a symmetrical Y maze (6). Thereafter they will be sacrificed to measure Aß concentrations and the number of amyloid plaques in the brain (6). My laboratory is well trained in the required immunohistology and ELISA techniques. Our in-house Morphology Unit will process and evaluate mouse brain sections to detect amyloid plaques by immunocytochemical staining and thioflavin S fluorescence (Fig. 3).

E. WORK PLAN—PAGE 5

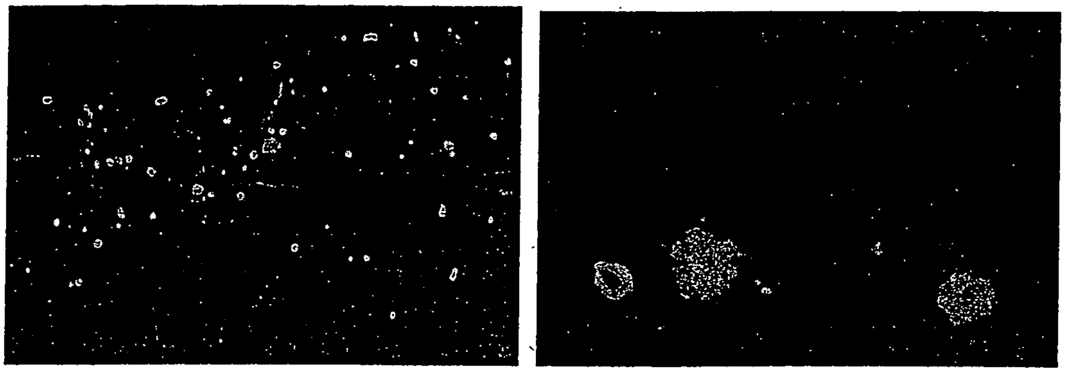

Fig. 3 Low and Hi Power Thioflavin Stained Brain Plaques from One of Our Tg Mice

If the long-term presence of high titre endogenous anti-Aß antibodies displaces Aß equilibria away from the brain, then transgenic mice from the group immunized with the Aß vaccine might show a delay or abrogation of symptoms. Compared to the non-specific control vaccine treated mice, these animals could have reduced concentrations of Aß in the brain and fewer or smaller amyloid plaques. If these changes in Aß do occur and the Aß-vaccinated animals also show improved memory and learning compared to controls, then the results would strengthen the correlation between soluble Aß, the plaques it forms and neurologic impairment.

In addition to testing the anti-Aß antibodies for preventing the formation of amyloid plaques and the development of memory deficits in young mice, we will also examine the effects of high titre anti-Aß on older transgenic mice which have plaques and are already neurologically affected. Experimental and control groups of old mice which were immunized starting when they were 12 months old will be tested as described above at the beginning of month 15 and on consecutive months thereafter.

These experiments with older mice are designed to test the possibility of reversing the symptoms in animals that already have elevated Aß and amyloid plaques in the brain as well as neurologic deficits. If the presence of high levels of anti-Aß antibodies displace Aß equilibria away from the brain then transgenic mice from the Aß-immunized group might show a reduction or abrogation of symptoms. Compared to the non-specific control antibody treated mice, the concentrations of Aß in the brain of these mice should be reduced and they might therefore have fewer or smaller amyloid plaques. If these changes in Aß do occur and the animals also show improved memory and learning compared to controls, then the results would strengthen the correlation between soluble Aß, the plaques it forms and neurologic impairment. If there is a significant reduction in amyloid plaques but no memory improvements then this might indicate either no causal relationship between plaques and neurologic deficits or simply that some secondary damage which had already done to the brain is irreversible. Throughout the course of these experiments the mice will be observed for any complications due to treatment with the Aß vaccines.

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6. K. Hsiao. et al.. *Science* **274**. 99-102 (1996)

F. AVAILABLE RESOURCES & BUDGET JUSTIFICATION

(Instructions: ctrl+alt F)

Resources: The PI occupies 1100 sq. ft. of laboratory and office space on the 2nd floor of the Boston Biomedical Research Institute and has access to 900 sq. ft. of common space. A complete small animal facility, including a room dedicated to breeding and handling transgenic mice, is located in the basement of the Institute.

BBRI has a Microvax and we are connected to the Internet. Several desktop computers are located in the lab and office for image processing, data analysis, literature searches, sequence analysis, etc. The Silicon Graphics Iris Indigo XS24 graphics workstation at BBRI is available to this project for molecular modeling.

BBRI has a well equipped machine shop, an in-house library and access to the Treadwell library at the Massachusetts General Hospital.

The BBRI has a PerSeptive Biosystems Voyager RP MALDI-TOF mass spectrometer. An in-house fluorescence flow cytometry facility is available. We have a molecular imager for acquiring and digitizing radioisotopic brain images.

An in-house Morphology Unit provides Histology Services, Electron Microscopy Services and Confocal Microscopy Services.

BBRI also has an X-ray crystallography facility.

Within the laboratory of the PI are: a scintillation counter, pH meter, semi-micro balance, top loading balance, Zeiss inverted tissue culture microscope, CO2 incubator, fraction collectors, UV monitor, LKB spectrophotometer, microscope, clinical centrifuge, 6 ft. laminar flow hood, 1 liquid N2 tank, flash evaporator, horizontal DNA/RNA gel electrophoresis apparatus, 2 microfuges, PAGE apparatus, a western blot apparatus, HPLC, and a video fluorescence microscope set-up.

Shared equipment at BBRI includes: a gamma counter, 3 scintillation counters, 4 ultracentrifuges, a peptide sequencer, 2 autoclaves, 3 cold rooms, 3 spectrophotometers, a DNA synthesizer, a peptide synthesizer, 3 PCR machines, 4 HPLC units, an electron microscope, a circular dichroism spectrophotometer, a florescence lifetime apparatus, a DU 650 spectrophotometer, a phosphoimager apparatus, and an ELISA plate reader.

Personnel: The principal investigator will devote 20% time/effort to supervise the overall project and the scientific endeavors of the research technician, Christine Kearney (50% time/effort). The P.I. and research technician will take responsibility for the production of ß-amyloid vaccines and their characterization. They will then evaluate these vaccines in a transgenic mouse model of Alzheimer's disease. The P.I. and research technician will:

- design, synthesize and purify several Aß peptides
- carry out mass spectral and amino acid analyses
- link Aß peptides to antigenic carriers
- perform genetic analyses on the transgenic "Alzheimer's mice"
- plan and implement immunization protocols using the Aß antigens in transgenic mice
- immunize young mice which have not yet developed cerebral plaques

F. AVAILABLE RESOURCES & BUDGET JUSTIFICATION- Page 2

- immunize old mice which have preestablished cerebral plaques
- screen immunized mice for the production of anti-Aß antibodies
- carry out radiolabeled-Aß biodistribution studies in immunized mice
- test anti-Aß antibodies for their ability to displace Aß equilibria in "Alzheimer's mice"
- examine vaccinated "Alzheimer's mice" for the effects of endogenous antibodies on the formation of plaques
- test the capacity of endogenous anti-Aß antibodies for delaying or reversing Aß plaque formation
- determine if anti-Aß antibodies will delay, prevent or reverse memory impairment in the "Alzheimer's mouse" model
- examine "Alzheimer's mice" for any adverse effects due to the production of anti-Aß antibodies
- run radioimmunoassays
- screen mouse sera by ELISA
- carry out learning and memory deficit evaluations
- order and maintain laboratory reagents and supplies

Supplies: The experimental studies planned will require supplies for the peptide synthesis needed to produce several different ß-amyloid antigens needed for these vaccine studies. Chemistry supplies and reagents are also needed for the purification and analysis of those peptides. Chromatography materials will be used for the isolation of both the new peptides and the antigens created after their conjugation to immunogenic carrier proteins. Immunochemicals are needed to perform the ELISA and radioimmunoassay protocols for monitoring antibody production in the transgenic mice. The purchase of animals for this project is also included in the supply costs.

Other Expenses: The breeding of transgenic mice and long term housing of the mouse colony is reflected in the animal costs. Publication costs are also requested.

Duration of Support: Three years of support have been requested. I believe that the productivity and pioneering discoveries of my laboratory has proven our long-term commitment to the study of immunology and to the development of new strategies for realizing its clinical usefulness. The proposed research is fairly straightforward but substantial time and effort will be required to achieve our goals. The duration requested will allow us to follow up on our preliminary findings and continue to gain new and important scientific insights. Moreover, the research has been designed so that its fundamental findings may have a direct application to Alzheimer's disease. I feel that the 3-year support is well justified for these reasons. Fringe benefits are calculated at 29% for professional and non-professional personnel.

G. COST ESTIMATE *(Instructions: ctrl+alt G)*

Categories	YEAR 1	YEAR 2	YEAR 3
Personnel			
Salaries: List here each member of the team plus % of time (FTE) allocated to the project			
Victor Raso (20%)	20,080	20,080	20,080
Chris Kearney (50%)	13,060	13,060	13,060
Benefits: List here each member of the team plus % of time (FTE) allocated to the project			
Victor Raso (20%)	5,823	5,823	5,823
Chris Kearney (50%)	3,787	3,787	3,787
Lab and Research (e.g. supplies including animals)	7,800	7,800	7,800
Consultants	0	0	0
Publication Costs	300	300	300
Travel (≤ $1000)	0	0	0
Subcontracts	0	0	0
Other	3,695	3,695	3,695
Total Direct Costs	54,545	54,545	54,545
Indirect Costs *Maximum of 10% of direct costs*	5,455	5,455	5,455
Total Direct & Indirect Costs	**60,000**	**60,000**	**60,000**
Cost Sharing			

H. APPLICANT INFORMATION: SCIENTIFIC, TECHNICAL AND ACADEMIC QUALIFICATIONS *(Instructions: ctrl+alt H)*

Principal Investigator: Victor A. Raso, Ph.D.

Professional Experience:

1973-1974	Associate in Pharmacology, Harvard Medical School
1973-1981	Research Associate, Division of Biochemical Pharmacology, Dana-Farber Cancer Institute
1981-1988	Assistant Professor of Pathology, Dana-Farber Cancer Institute, Harvard Medical School
1988-1989	Principal Scientist, Boston Biomedical Research Institute
1989-	Senior Scientist, BBRI, Dept. of Cell & Molecular Biology

Publications (Partial):

1. Raso, V and Stollar, BD. Antibodies specific for conformationally distinct coenzyme-substrate transition state analogues. A fluorescence, N.M.R., circular dichroism and antibody study of N-(5-phosphopyridoxyl)-3'-amino-L-tyrosine. J. Amer. Chem. Soc. 1973; 95:1621.
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KRS: *APPLICATION FORM FOR INDIVIDUAL RESEARCH PROJECT©*
Application Number *(for office use only)*:
Project Title:

I. APPLICANT INFORMATION: LIST OF CONFLICTS

(Instructions: ctrl+alt I)

REVIEWER CONFLICTS *(Use Font Size 8)*				
Name	Address	Phone	FAX	E-Mail

KRS: *APPLICATION FORM FOR INDIVIDUAL RESEARCH PROJECT*©
Application Number *(for office use only)*:
Project Title:

J. APPLICANT INFORMATION: TECHNICAL REVIEWERS
(Instructions: ctrl+alt J)

REVIEWERS: *Please provide 15 individual contacts (Use Font Size 8)*				
Name	Address	Phone	FAX	E-Mail
Dr. R. E. Tanzi	Dept. Neurol., Massachusetts Gen. Hosp., Charlestown, MA 02129	(617) 726-6845	(617) 726-5736	tanzi@helix.mgh.harvard.edu
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Dr. Lawrence Recht	Dept. Neurol., U Massachusetts Med Center, 55 Lake Ave, Worcester, MA 01655	(508) 856-4147	(508) 856-6778	lawrence.recht@banyon.ummed.edu

Keyword List

(Type of) Glutamate Receptor
Acetylcholine Anatomy /
Activities of Daily Living
Adeenergic System
Adult Day Care
Aluminum
Amyloid Diseases
Amyloid Precursor Protein
Anatomy
Animal Behavior
Animal models
Animal Models-Trangenics
Antidepressants
Antisense Oligonucleotides
Anxiety
Apolipoprotein
Apoptosis
Autonomic mechanisms
Axonal Transport
Behavioral Effects
Behavioral Management
Behavioral Neurology
Behavioral Pharmacology
Beta-amyloid
Biochemistry
Biological Assays
Blood Brain Barrier
Calcium Imaging
Caregiving Issues
Cell Biology
Cell Cultures
Cell Death
Cellular Physiology
Cerebral Metabolism
Cerebrospinal Fluid
Cholinergic System
Circadian rhythms
Clinical Assessment
Clinical Care
Clinical Diagnosis
Clinical Pharmacology
Clinical Trials

Cloning
Cognition
Cognitive Neuroscience
Cognitive Psychology
Cognitive Therapy
Communication/Language
Cost Benefit Analysis
Cytoskeleton
Dentistry
Depression
Developmental Neurobiology
Disabilities
DNA Sequencing
Down Syndrome
Drosophila Neurobiology
Drug Delivery
Drug Design
Drug Metabolism
Elder Abuse
Electroencephalography
Electron Microscopy
Electrophysiology
Endocrinology
Enzyme linked immunosorbent
Enzymology
Epidemiology
Estrogen
Ethical Issues
Event related potential
Excitotoxins
Familial Alzheimer's Disease
Forebrain
Free Radicals
GABA Receptors
Gangliosides
Gene Cloning
Gene Expression
Gene Mapping
Gene Transfer
Genetic Linkage
Genetics
Gerontology

Glutamate
Glutamate Receptors
Glycoproteins
Growth Factors
Health Care Financing
Health Facilities & Services
Health Financing
Hemostasis
Herpes Simplex Virus
Hippocampus
Histochemistry
Histopathology
Human Genetics
Immunoassay
Immunocytochemistry
Immunohistochemistry
Immunology
In Situ Hybridization
In Vitro Methods
In Vivo Microdialysis
Instrument Development
Ion Channels
Language
Legal Issues
Lewy Bodies
Linkage Analysis
Lipid Metabolism
Long Term Potentiation
Longitudinal Study
Magnetic Reson.
Magnetic Resonance Imaging
magnetoencephalography
Mass Spectroscopy
Medicinal Chemistry
Medicine
Membrane Biochemistry
Memory
Messenger RNA
Microtubules
Microvessels
Molecular Biology
Molecular Genetics
Morphology

Movement Disorders
MR spectroscopic imaging
Multicultural Issues
Muscarinic Receptors
Nerve Growth Factor
Neural Degeneration
Neural Development
Neural System
Neural System-Olfactory
Neural System-Visual
Neural Transplantation
Neural-Viral Interactions
Neuroanatomy
Neurobiology
Neurochemistry
Neuroendocrinology
Neurofibrillary Tangles
Neurofilaments
Neuroimmunology
Neurology
Neuromorphology
Neuronal Cell Death
Neuroopthalmology
Neuropathology
Neuropharmacology
Neurophysiology
Neuropsychological Testing
Neuropsychology
Neurosurgery
Neurotoxicology
Neurotrophic Factors
Nicotine
Non-Cognitive Behavior
Normal Aging
Northern Blot Analysis
Nuclear Magnetic Resonance
Nursing
Nursing Home Research
Nutrition
Oral Health
Otolaryngology
Oxidative Injury
Paired Helical Filaments

KRS: ***APPLICATION FORM FOR INDIVIDUAL RESEARCH PROJECT©***
Application Number *(for office use only)*:
Project Title:

Parkinson's Disease	Surveys
Patch Clamp	Tau Protein
Pathology	Tissue Cultures
Patient Care	Trace Elements
Pharmacokinetics	Transgenic Mice
Pharmacology	Trisomy
Physical Chemistry	Trophic Factors
Physiological Psychology	Vascular Disease
Physiology	Videotaping
Plaques	Virology
Policy Analysis	Vision
Polymerase chain reaction	Visuospatial Attention
Positron Emission	Voltage Clamp
Presymptomatic Testing	
Primates	
Prion Proteins	
Program Evaluation	
Proteases	
Psychiatry	
Psychology	
Psychopharmacology	
Qualitative Methods	
Quantitative Autoradiography	
Quantitative RNA/DNA	
Radiology/Neuroimaging	
Radiopharmaceuticals	
Receptors	
Regeneration (Sprouting)	
Respite Care	
Risk Factors	
RNA Analysis	
Second Messengers	
Senescence	
Signal Transduction	
Single Unit Recording	
Sleep	
Social Work	
Sociology	
SPECT-Single Photon Emis,	
Staffing/Training Issues	
Staging of Dementia	
Statistical Procedures	
Surgery	

Expiration Date 2/28/98

Department of Health and Human Services
Public Health Service
Small Business Innovation Research Program

Phase I Grant Application

Follow instructions carefully.

Leave blank — for PHS use only.		
Type	Activity	Number
Review Group		Formerly
Council Board *(Month, year)*		Date Received

1. TITLE OF APPLICATION *(Do not exceed 56 typewriter spaces)*
Bispecific Probe to Visualize Intracellular Beta-Amyloid

2. SOLICITATION NO. PHS 97-2 Probes and Instruments for Micro-Imaging the Brain PA-99-007

3. PRINCIPAL INVESTIGATOR ☐ New Investigator

3a. NAME *(Last, first, middle)*: RASO, VICTOR A.

3b. DEGREE(S): B.S | Ph.D.

[redacted]

3d. POSITION TITLE: SENIOR SCIENTIST

3e. MAILING ADDRESS *(Street, city, state, zip code)*
BOSTON BIOMEDICAL RES. INST.
20 STANIFORD STREET
BOSTON, MA 02114

3f. TELEPHONE AND FAX *(Area code, number, and extension)*
TEL: (617) 912-0316
FAX: (617) 912-0308

BITNET/INTERNET Address:

4. HUMAN SUBJECTS
[X] NO
☐ YES

4a. If "yes," Exemption no. or IRB approval date ☐ Full IRB or ☐ Expedited Review

4b. Assurance of compliance no.

5. VERTEBRATE ANIMALS
☐ NO
[X] YES

5a. If "Yes," IACUC approval date: 4/27/98

5b. Animal welfare assurance no.: A3177-02

6. DATES OF PROJECT PERIOD
From: 10/01/99 Through: 03/31/01

7. COSTS REQUESTED
7a. Direct Costs $ 400,000
7b. Total Costs $ 400,000

8. PERFORMANCE SITES *(Organizations and addresses)*
BOSTON BIOMEDICAL RESEARCH INSTITUTE
20 STANIFORD STREET
BOSTON, MA 02114

9. APPLICANT ORGANIZATION *(Name and address of applicant small business concern)*
BOSTON BIOTECHNOLOGY CORP.
20 STANIFORD STREET
BOSTON, MA 02114

10. ENTITY IDENTIFICATION NUMBER: 1042766443A1
Congressional District: 9

11. SMALL BUSINESS CERTIFICATION
[X] Small Business Concern ☐ Women-owned
☐ Socially and Economically Disadvantaged

12. NOTICE OF PROPRIETARY INFORMATION: The information identified by asterisks(*) on pages ________ of this application constitutes trade secrets or information that is commercial or financial and confidential or privileged. It is furnished to the Government in confidence with the understanding that such information shall be used or disclosed only for evaluation of this application, provided that, if a grant is awarded as a result of or in connection with the submission of this application, the Government shall have the right to use or disclose the information herein to the extent provided by law. This restriction does not limit the Government's right to use the information if it is obtained without restriction from another source.

14. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name: THOMAS McQUAID
Title: ASSISTANT TREASURER
Address: 20 STANIFORD STREET
BOSTON, MA 02114

Telephone: (617) 912-0301
FAX: (617) 912-0335
BITNET/INTERNET Address:

13. DISCLOSURE PERMISSION STATEMENT: If this application does not result in an award, is the Government permitted to disclose the title only of your proposed project, and the name, address, and telephone number of the official signing for the applicant organization, to organizations that may be interested in contacting you for further information or possible investment? ☐ YES ☐ NO

15. PRINCIPAL INVESTIGATOR ASSURANCE: I certify that the statements herein are true, complete, and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PERSON NAMED IN 3a *(In ink. "Per" signature not acceptable.)*
Victor Raso
DATE 4/12/99

16. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete, and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF PERSON NAMED IN 14 *(In ink. "Per" signature not acceptable.)*
Thomas McQuaid
DATE 4/12/99

B000450

AA

Form Approved Through 9/30/97
OMB No. 0925-0001

Department of Health and Human Services
Public Health Service

Grant Application

Follow instructions carefully.
Do not exceed character length restrictions indicated on sample.

LEAVE BLANK-FOR PHS USE ONLY.		
Type	Activity	Number
Review Group	Formerly	
Council/Board (*Month, Year*)		Date Received

1. TITLE OF PROJECT (*Do not exceed 56 characters, including spaces and punctuation.*)
CEREBRAL DELIVERY OF VECTORIZED ANTI-BETA-AMYLOID ANTIBODY

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT ☐ NO ☒ YES (*If "Yes," state number and title*)
Number: Title: PREPARED IN RESPONSE TO NIA AND NINDS ANNOUNCEMENT--ALZHEIMER'S DISEASE

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR

3a. NAME (*Last, first, middle*)
RASO, VICTOR A.

3b. DEGREE(S)
PH.D.

3c. SOCIAL SECURITY NO.

3d. POSITION TITLE
SENIOR SCIENTIST

3e. MAILING ADDRESS (*Street, city, state, zip code*)
BOSTON BIOMEDICAL RESEARCH INST.
20 STANIFORD STREET
BOSTON, MA 02114-2500

3f. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT

3g. MAJOR SUBDIVISION

3h. TELEPHONE AND FAX (*Area code, number and extension*)
TEL: (617) 912-0316
FAX: (617) 912-0308

E-MAIL ADDRESS:
RASO@BBRI.HARVARD.EDU

4. HUMAN SUBJECTS ☒ No ☐ Yes

4a. If "Yes," Exemption no. or IRB approval date ☐ Full IRB or ☐ Expedited Review

4b. Assurance of compliance no.

5. VERTEBRATE ANIMALS ☐ No ☒ Yes

5a. If "Yes," IACUC approval date: 02/02/96

5b. Animal welfare assurance no.: A3177-01

6. DATES OF PROPOSED PERIOD OF SUPPORT (*month, day, year-MM/DD/YY*)		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)
7/01/99	6/30/03	$198,885	$348,944	$829,005	$1,466,226

9. APPLICANT ORGANIZATION
Name
Address
BOSTON BIOMEDICAL RESEARCH INST.
20 STANIFORD STREET
BOSTON, MA 02114-2500

10. TYPE OF ORGANIZATION
Public: → ☐ Federal ☐ State ☐ Local
Private: → ☒ Private Nonprofit
Forprofit: → ☐ General ☐ Small Business

11. ORGANIZATIONAL COMPONENT CODE 60

12. ENTITY IDENTIFICATION NUMBER 1042451939A1

Congressional District 9

13. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
Name THOMAS J. MCQUAID
Title ASSISTANT DIRECTOR
Address BOSTON BIOMEDICAL RESEARCH INST.
20 STANIFORD STREET
BOSTON, MA 02114-2500
Telephone (617) 912-0301
FAX (617) 912-0335
E-Mail Address MCQUAID@BBRI.HARVARD.EDU

14. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name KATHLEEN G. MORGAN, PH.D
Title DIRECTOR
Address BOSTON BIOMEDICAL RESEARCH INST.
20 STANIFORD STREET
BOSTON, MA 02114-2500
Phone (617) 912-0330
FAX (617) 227-6053
E-Mail Address MORGAN@BBRI.HARVARD.EDU

15. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PI/PD NAMED IN 3a. (*In ink. "Per" signature not acceptable.*)
Victor Raso

DATE 10/11/98

16. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 14. (*In ink. "Per" signature not acceptable.*)
Kathleen G Morgan

DATE 10/15/98

…RIPTION. State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely …e research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This description is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. **DO NOT EXCEED THE SPACE PROVIDED.**

The ß-amyloid peptide and the cerebral plaques that it forms are likely either the direct or indirect cause of Alzheimer's disease. This small, 43 amino acid residue peptide is produced in the central nervous system by cleavage from a cell-surface precursor protein. Thus, soluble ß-amyloid exists free in the brain and cerebrospinal fluid and it eventually deposits as "insoluble" aggregates to form amyloid plaques in the brain. The hypothesis to be tested suggests that the soluble and insoluble forms of ß-amyloid present within the brain of Alzheimer's patients are in dynamic equilbrium. Accordingly, plaque growth will be curtailed and the plaques should gradually dissolve when that equilibrium is displaced by reducing soluble ß-amyloid levels. This depletion can be accomplished by using highly specific anti-ß-amyloid antibodies to tie up or modify soluble ß-amyloid in the brain. Initially, these reagents will be infused intracerebrally into cannulated mice. Transport of the anti-ß-amyloid antibody into the central nervous system will also be facilitated by coupling it to an anti-transferrin receptor antibody which serves as a vector, carrying the new bispecific construct across the blood-brain barrier. These expressly designed, vectorized anti-ß-amyloid antibodies provide a novel basis for the immunotherapy of Alzheimer's disease by virtue of their ability to enter the brain and directly perturb ß-amyloid equilibria.

The effect of those vectorized antibodies will be tested using an established colony of Tg2576 transgenic mice which express a mutant form of the human amyloid precursor protein and produce extracellular ß-amyloid peptide deposits in the brain at ~11 months of age. Human ß-amyloid peptides and transition state analogs of those peptides have been used to elicit monoclonal antibodies in mice. The antibodies have been chacterized by ELISA and proteolytic assays to select those that show high-affinity binding to ß-amyloid, or catalytic activity, or an ability to dissolve ß-amyloid aggregates. Bispecific antibodies were formed by coupling the anti-ß-amyloid antibodies to an anti-transferrin receptor antibody which can cross the blood-brain barrier by transcytosis. These vectorized bispecific antibodies will be administered to young and old transgenic mice by periodic i.p. injection either before or after the onset of plaque development. Antibody-treated transgenic mice will be compared to control transgenic mice in terms of the number and size of plaques in brain sections, the level of ß-amyloid peptides in brain extracts and the extent of their memory impairment. These studies could establish a causal relationship between amyloid deposits and the behavioral deficits in the transgenic mouse model.

PERFORMANCE SITE(S) *(organization, city, state)*

BOSTON BIOMEDICAL RESEARCH INST
20 STANIFORD STREET
BOSTON, MA 02114

KEY PERSONNEL. See instructions on Page 11. *Use continuation pages as needed* to provide the required information in the format shown below.

Name	Organization	Role on Project
Victor Raso	Boston Biomedical Research Institute	Principal Investigator
Katherine Sheldon	Boston Biomedical Research Institute	Research Associate
Christine Kearney	Boston Biomedical Research Institute	Research Technician

RESEARCH GRANT

TABLE OF CONTENTS

*Type density and type size of the entire application must conform to limits provided in instructions on page 6.

Appendix *(Five collated sets. No page numbering necessary for Appendix)* — X Check if Appendix is Included

Number of publications and manuscripts accepted or submitted for publication *(not to exceed 10)* 6

Other items *(list)*:

Figure 7
Figure 14
Figure 18
Figure 19

Four Letters of Support

Principal Investigator/Program Director (Last, first, middle): Raso, Victor A.

DETAILED BUDGET FOR INITIAL BUDGET PERIOD DIRECT COSTS ONLY

FROM 12/1/98 THROUGH 11/30/02

PERSONNEL (Applicant organization only)

NAME	ROLE ON PROJECT	TYPE APPT. (months)	% EFFORT ON PROJ.	INST. BASE SALARY	SALARY REQUESTED	FRINGE BENEFITS	TOTALS
Victor A. Raso	Principal Investigator	12.0	50	$104,650	$52,325	$15,174	$67,499
Katherine Sheldon	Res Assoc	12.0	100	$42,950	$42,950	$12,456	$55,406
Christine Kearney	Res Tech	12.0	100	$27,515	$27,515	$7,979	$35,494
Angela J. DiPerri	Admin. Assist.	12.0	10	$36,500	$3,650	$1,059	$4,709
SUBTOTALS					$126,440	$36,668	$163,108

CONSULTANT COSTS

EQUIPMENT (Itemize)

Liquid Nitrogen Tank	$3,338	
DEAE memsep column	$1,500	
ProA memsep column	$575	
12-channel pipetter	$650	$6,063

SUPPLIES (Itemize by

Tissue culture sup. (media,sera,etc.)	$7,500	Chromatography matls.	$2,500	
Glassware & Disposables	$1,500	Chemistry sup./reagents	$2,500	
Consumable Lab Supplies	$2,000	100 mice @ 7.14	$714	
Radiochemicals	$1,500			
Immunochemicals	$2,000			$20,214

TRAVEL

Conference — $1,500

PATIENT CARE COSTS — INPATIENT / OUTPATIENT

ALTERATIONS AND RENOVATIONS (Itemize by category)

OTHER EXPENSES (Itemize by category)

Intracerebral Cannulation	$500	
Publ. Costs	$1,000	
Animal Housing/Breeding	$6,000	
Histological Services	$500	$8,000

SUBTOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD $ 198,885

CONSORTIUM/CONTRACTUAL COSTS — DIRECT COSTS / INDIRECT COSTS

TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD (Item 7a, Face Page) → $ 198,885

BUDGET FOR ENTIRE PROPOSED PERIOD OF SUPPORT
DIRECT COSTS ONLY

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD (from Form Page 4)	ADDITIONAL YEARS OF SUPPORT REQUESTED 2nd	3rd	4th	5th
PERSONNEL: *Salary and fringe benefits Applicant organization only*		$163,108	$169,632	$176,418	$183,474	
CONSULTANT COSTS						
EQUIPMENT		$6,063	$1,500	$1,500	$1,500	
SUPPLIES		$20,214	$21,023	$21,863	$22,738	
TRAVEL		$1,500	$1,500	$1,500	$1,500	
PATIENT CARE COSTS	INPATIENT					
	OUTPATIENT					
ALTERATIONS AND RENOVATIONS						
OTHER EXPENSES		$8,000	$8,320	$8,653	$8,999	
SUBTOTAL DIRECT COSTS		$198,885	$201,975	$209,934	$218,211	
CONSORTIUM/ CONTRACTUAL COSTS	DIRECT					
	INDIRECT					
TOTAL DIRECT COSTS		$198,885	$201,975	$209,934	$218,211	

TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PERIOD OF SUPPORT *(Item 8a, Face Page)* → $ 829,005

JUSTIFICATION. Follow the budget justification instructions exactly. Use continuation pages as needed.

Personnel: The P.I. will devote 50% time/effort to supervise the overall project and the scientific endeavors of both the res. assoc., Katherine Sheldon (100% time/effort) and the res. tech., Christine Kearney (100% time/effort). The P.I. and res. assoc. will take responsibility for the chemical synthesis and evaluation of all transition state peptides, their conjugation to antigenic carriers, as well as production of monoclonal antibodies and their characterization. They will

- design and synthesize several Aß and transition state analog Aß peptides
- carry out mass spectral analyses
- perform energy and conformational analyses of transition state vesus native Aß peptides on graphics workstation
- plan and implement immunization protocols for each of the many hybridoma fusions required in this project
- perform hybridoma fusions to generate anti-Aß and anti-transition state analog Aß peptide antibodies
- screen the specificity of these new anti-Aß monoclonal antibodies
- develop and utilize catalytic antibody screening assays based upon hydrolytic cleavage of the Aß peptide
- purifiy antibodies to fully define their specificity and catalytic activity
- screen the these new anti-Aß monoclonal antibodies for their ability to dissolve Aß aggregates
- analyze the peptide cleavage products produced by the catalytic antibodies
- carry out radiolabeled-Aß biodistribution studies in mice
- perform genetic analyses on "Alzheimer's mice"
- determine if i.v. administered radiolabeled-Aß will localize within the plaques of "Alzheimer's mice"
- perform intracerebral injections of anti-Aß antibodies into cannulated Tg mice
- construct the different vectorized anti-Aß reagents needed for this project
- compare different vectorized antibodies for their ability to displace Aß equilibria in "Alzheimer's mice"
- determine the ability of passively administered vectorized anti-Aß antibodies to delay or reverse Aß plaque formation
- determine if vectorized anti-Aß antibodies will delay or prevent memory impairment in the "Alzheimer's mouse"
- examine "Alzheimer's mice" for adverse effects due to treatment with vectorized anti-Aß antibodies

A full time research technician is required to maintain the smooth operation of all of the support functions which allow the laboratory to run effectively. Some of the technical areas for this project include:

- collection of ascites from mice for monoclonal antibody isolation
- the purification and analysis of peptides by HPLC
- the analysis of peptide conjugates by PAGE
- determination of amino acid composition of the Aß and Aß transition state analog antigens
- maintenance of established cell lines and hybridoma clones
- bleeding mice to test for the production of antibodies
- running radioimmunoassays
- assisting in the performance of hybridoma fusions
- cryo-preservation of cells
- screening hybridoma clones by ELISA
- collection of ascites from mice for monoclonal antibody isolation
- purification and testing of monoclonal antibodies
- assisting in the brain imaging studies
- assisting in the learning and memory deficit evaluations
- ordering and maintaining laboratory reagents and supplies
- sterilization of equipment, cages and surgical instruments
- preparation of buffers and reagents

Administrative assistance is needed for typing manuscripts and correspondence as well as for maintaining records of purchases and expenditures.

Equipment: A variable volume 12-channel pipetter is needed for removing, transferring and dispensing small aliquots of hybridoma supernatant from multiple 96-well plates during catalytic antibody screening procedures. The protein A Memsep and DEAE Memsep columns are needed to prepare highly purified monoclonal antibodies and Fab fragments which must be free of extraneous enzymatic activity. The liquid nitrogen freezer is requested because we have exceeded the storage capacity of our present freezer and we are producing new hybridomas at a prodigious rate.

Supplies: The experimental studies planned will require substantial supplies for each of its distinct components; peptide synthesis, hybridoma production, monoclonal antibody purification, bispecific antibody synthesis, transgenic mouse biodistribution and therapy trials. The generation of numerous monoclonal antibodies is reflected in the need for purchasing adequate tissue culture supplies and for the considerable number of mice required for immunization and ascites production. The chemistry supplies are needed for the synthesis of all of the native and transition state peptides required for inducing and testing anti-Aß antibodies. The funds requested include the cost of F-moc-statine analogs (~$1,000/gm) that are required for several of the transition state peptides described in the proposal. Chromatography materials will be used during the synthesis of new peptides and for the purification of monoclonal antibodies.

Other Expenses: Intracerebral cannulation of Tg mice will be performed by Tacconic Laboratories at a cost of ~ $38/mouse. Animal housing is requested for maintenance of the transgenic mice and mice used in the numerous hybridoma fusions which will be performed and for ascites production. Funds for travel to one or two national meetings per year are requested for both the P.I. and research associate to present research findings.

Duration of Support: Four years of support have been requested. I believe that the productivity and pioneering discoveries of my laboratory have proven our long-term commitment to the study of immunology and to the development of new strategies for realizing its clinical usefulness. The proposed research is fairly straightforward but many problems will have to be addressed to achieve our goals. The duration requested will allow us to follow up these novel developments and continue to gain new and important scientific insights. Moreover, the research has been designed so that its fundamental findings may have a direct application to human disease. I feel that the 4-year support is well justified for these reasons.

All categories are projected at a 4% increase per year. Equipment is projected at $1,500 for years 2-4 for the purchase of small equipment items to replace worn or obsolete instruments as needed. Fringe benefits are calculated at 29% for professional and non-professional personnel. Indirect costs are calculated at 92% based on salary and wage.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Photocopy this page or follow this format for each person

NAME	POSITION TITLE
Victor A. Raso	Sr. Scientist

EDUCATION/TRAINING (*Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.*)

INSTITUTION AND LOCATION	DEGREE (*if applicable*)	YEAR(s)	FIELD OF STUDY
Fordham University, Bronx, NY	B.S.	1967	Biology
Tufts University, Boston, MA	Ph.D.	1973	Biochemistry Immunochemistry

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. DO NOT EXCEED TWO PAGES.

Professional Experience:

1973-1974	Associate in Pharmacology, Harvard Medical School
1973-1981	Research Associate, Division of Biochemical Pharmacology, Dana-Farber Cancer Institute
1981-1988	Assistant Professor of Pathology, Dana-Farber Cancer Institute, Harvard Medical School
1988-1989	Principal Scientist, Boston Biomedical Res Inst., Dept. of Cell & Mol Biology
1989-	Senior Scientist, BBRI

Honors:

1983	Invited Lecturer and Tutor at the NATO Advanced Studies Inst., "Receptor Mediated Targeting of Drugs," Cape Sounion, Greece
1983	Lecturer, "Workshop on the Use of Monoclonal Antibodies in Tumor Therapy," National Institutes of Health, Bethesda, MD.
1984	Invited Lecturer at the American Association for the Advancement of Science Symposium on "Molecular Targeting of Drugs," NY, NY
1984	Invited Speaker at the Gordon Research Conference on "Drug Carriers in Biology and Medicine," Plymouth, NH
1986	Invited Lecturer at the Plenary Sess. in the UCLA Sympos. on Molecular and Cellular Biology, "Membrane-Mediated Cytotoxicity," Park City, UT
1986	Invited Speaker, Gordon Conference on "Drug Carriers in Biology and Medicine," Plymouth, NH
1987	Symposium Speaker, "Biological Approaches to the Controlled Delivery of Drugs: Barriers, Technology and Therapies," New York Acad. of Sciences, NY
1988	Session Chairman at the First Internat. Symp. on Immunotoxins, Durham, NC
1988	Recipient of the Pierce Immunotoxin Award
1990	Speaker at Second Internat. Sympos. on Immunotoxins, Buena Vista, FL.
1990	Chairman and Speaker, Fifth International Conference on Monoclonal Antibody Immunoconjugates for Cancer, San Diego, CA
1993	Invited to lecture at the American Association for the Advancement of Science Symposium "Drug Targeting in Diagnosis and Therapy," Boston
1996	Chairman and Speaker, Exploring and Exploiting Antibody and Ig Superfamily Combining Sites, Novel Immunotoxin Strategies, Keystone Symposia, Taos, NM

Publications (Partial):

1. Raso, V and Stollar, BD. Antibodies specific for conformationally distinct coenzyme-substrate transition state analogues. A fluorescence, N.M.R., circular dichroism and antibody study of N-(5-phosphopyridoxyl)-3'-amino-L-tyrosine. J. Amer. Chem. Soc. 1973; 95:1621.

2. Raso V and Stollar BD. The antibody-enzyme analogy. Characterization of antibodies to phosphopyridoxyltyrosine derivatives. Biochem. 1975; 14: 584-591.
3. Raso V and Stollar BD. The antibody-enzyme analogy. Comparison of enzymes and antibodies specific for phosphopyridoxyltyrosine. Biochem. 1975; 14: 591-599.
4. Raso V and Griffin T. Specific cytotoxicity of a human immunoglobulin directed Fab'-ricin A chain conjugate. J Immunol. 1980; 125 :2610.
5. Raso V and Griffin T. Hybrid antibodies with dual specificity for the delivery of ricin to immunoglobulin bearing target cells. Cancer Res. 1981; 41:2073.
6. Raso V, Ritz, J. Basala M and Schlossman SF. A monoclonal antibody-ricin A chain conjugate which is selectively cytotoxic for cells bearing the common acute lymphoblastic leukemia antigen (CALLA). Cancer Res. 1982; 42:457.
7. Raso V. Antibody mediated delivery of toxin molecules to antigen bearing target cells. In: Moller, G, Ed. Immunological Reviews: Antibody carriers of drugs and toxins in tumor therapy. Copenhagen: Munksgaard, 1982: 93-117.
8. Raso V and Basala M. A highly cytotoxic human transferrin-ricin A chain conjugate used to select receptor modified cells. J Biol Chem. 1984; 259:1143.
9. Raso V and Lawrence J. Carboxylic ionophores enhance the cytotoxic potency of ligand- and antibody-delivered ricin A chain. J Exp Med. 1984; 160:1234.
10. Raso, V, Watkins SC, Slayter H and Fehrmann C. Intracellular pathways of ricin A chain cytotoxins. In: Juliano R, Ed. Biological approaches to the controlled delivery of drugs: barriers, technologies and therapies. Ann. of the New York Acad. of Sci.. 1988; 507:172-185.
11. Raso V. and McGrath J. Diphtheria toxin cures athymic mice of human malignant mesothelioma. J. Natl. Cancer Inst. 1989; 81, 622-627.
12. Raso, V. The magic bullet - nearing the century mark. In: Osborn, M. ed. Seminars in Cancer Biology. Antibodies in diagnosis and therapy, 1990. vol. 1 pp 227-242
13. Recht, L., Torres, C. O., Smith, T. W., Raso, V. A., and Griffin, T. W. (1990) Transferrin receptor in normal and neoplastic brain tissue: Implications for brain-tumor immunotherapy. *J Neurosurg* 72, 941-945.
14. Rakowicz-Szulczynska, E, Kaczmarski, W, Raso, V, Steimer, KS, Durda, P. Internalization of anti-gp120 monoclonal antibody and human antibodies by HIV-1-infected T lymphocytes. Antibody, Immunoconjugates, and Radiopharmaceuticals 1993; 6: 209-219
15. Griffin, T. and Raso, V. Monensin in lipid emulsion for the *in vivo* potentiation of ricin A chain immunotoxins. Cancer Res., 1991;51:4316-4322.
16 Griffin, T., Rybak, M.E., Recht, L., Singh, M., Salimi, A., Raso, V. Potentiation of antitumor immunotoxins by liposomal monensin. J. Natl. Cancer Inst. 1993; 85: 292-298.
17. Raso, V. Immunotargeting Intracellular Compartments. Anal. Biochem. 1994; 222: 294-304.
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BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Photocopy this page or follow this format for each person

NAME	POSITION TITLE
Katherine M. Sheldon	Research Associate

EDUCATION/TRAINING (*Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.*)

INSTITUTION AND LOCATION	DEGREE (*if applicable*)	YEAR(s)	FIELD OF STUDY
Acadia University, Canada	BScH	1985	Chemistry
Univ. of Toronto, Ont., Canada	MSc	1987	Biochemistry
	PhD	1991	Biochemistry

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. DO NOT EXCEED TWO PAGES.

Professional Experience:

1991-1993	Postdoctoral fellow, Ontario Cancer Institute, Toronto, Ont., working on production of novel peptides that can be used as cellular targeting agents.
1993 - present	Research Associate, Boston Biomedical Research Inst., Boston, MA. Immunochemistry and monoclonal antibody development

Publications:

Baumal, R., Law, J., Buick, R., Kahn, H., Sheldon, K., Colgan, T., and Marks, A. Monoclonal antibodies to an epithelial ovarian adenocarcinoma: Distinctive reactivity with xenografts of the original tumour and cultured cell line. 1986. *Cancer Res.*46:3994-4000.

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Ettenson, D., Sheldon, K., Marks, A., Houston, L. and Baumal, R. Comparison of growth inhibition of a human ovarian adenocarcinoma cell line by free monoclonal antibodies and their corresponding antibody recombinant ricin A immunotoxins. 1988. *Anticancer Res.* **8**:833-838.

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Sheldon, K. and Sheldon, R.W. A new technique for detecting fluorescently labelled cells at very low densities. (1993) *Anticancer Res.* **13**:459-466.

Texic, M., Sheldon, K.M., Ballinger, J.R., and Boxen, I. Labelling small quantities of monoclonal antibodies and their F(ab')2 fragments with technetium-99m. (1995) *Nucl. Med. Biol.* 22: 451-457.

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Remy, S., Reilly, R., Sheldon, K., and Gariepy, J. A new radioligand for the epidermal growth factor receptor: 111In labelled human epidermal growth factor derivatized with a bifunctional metal-chelating peptide. (1995) *Bioconj. Chem.* 6: 683-690.

Sheldon, K., Liu, D., Ferguson, J., and Gariepy, J. Oligomers: design of *de novo* peptide-based intracellular vehicles. (1995) *Proc. Natl. Acad. Sci. USA* 92: 2056-2060.

OTHER SUPPORT

Raso, V.
PENDING
NIH (Raso) 4/1/99-3/31/01
$70,000 30%

A Binary System for Cell-Targeted Delivery
The major goal of this project is to develop a two part system for delivering the isolated, non-toxic, complementary functional domains of a toxin to two distinct target sites on a cell. Improved targeting of cells and reduced non-specific toxicity will result because these non-toxic halves can combine to regain activity exclusively in the dual-labeled target cells.
OVERLAP
There is no scientific overlap between the application under consideration and this proposal which was submitted. There is budgetary overlap so if both are funded my percent effort would be adjusted and additional personnel would be recruited to fulfill the objectives.

Raso, V.
PENDING
NIH (Raso) 4/1/99- 3/31/02
$90,000 30%

Vaccine to Elicit Catalytic Anti-Cocaine Antibodies
The major goal of this project is to develop a vaccine which will induce catalytic antibodies capable of inactivating cocaine by hydrolytic cleavage.
OVERLAP
There is no scientific overlap between the application under consideration and this proposal which was submitted. There may be some budgetary overlap so if both are funded my percent effort would be adjusted appropriately in conjunction with agency staff.

Raso, V.
PENDING
NIH (Raso) 4/1/99- 3/31/02
$150,000 30%

Vaccine to Elicit Catalytic Antibodies Against HIV
The major goal of this project is to develop catalytic antibodies that will inactivate HIV by specifically recognizing and catalytically cleaving its gp120 viral envelope protein.
OVERLAP
There is no scientific overlap between the application under consideration and this proposal which was submitted. There may be some budgetary overlap so if both are funded my percent effort would be adjusted appropriately in conjunction with agency staff.

Raso, V.
PENDING
NIH (Raso) 4/1/99- 3/31/01
$75,000 30%

Vaccine To Modulate Systemic ß-Amyloid Levels
The major goal of this project is to develop a vaccine to induce conventional, systemic antibodies directed against the ß-amyloid peptide for potential use in the immunotherapy of Alzheimer's disease.
OVERLAP
There is minor scientific overlap between the application under consideration and this proposal which was submitted. There may be some budgetary overlap so if both are funded my percent effort would be adjusted appropriately in conjunction with agency staff.

Raso, V.
PENDING
Alzheimer's Association (Raso) 6/30/99-5/31/02
$60,000 20%

Vaccine To Modulate Systemic ß-Amyloid Levels
The major goal of this project is to develop a vaccine to induce conventional, systemic antibodies directed against the ß-amyloid peptide for potential use in the immunotherapy of Alzheimer's disease.
OVERLAP
There is minor scientific overlap between the application under consideration and this proposal which was submitted. There may be some budgetary overlap so if both are funded my percent effort would be adjusted appropriately in conjunction with agency staff.

RESOURCES

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory: The PI occupies approximately 1000 sq. ft. of laboratory space on the 2nd floor of the Boston Biomedical Research Institute and has access to 900 sq. ft. of common space.

Clinical:

Animal: A complete small animal facility, including a room dedicated to handling athymic and transgenic mice, is located in the basement of the Institute.

Computer: BBRI has a Microvax and we are connected to the Internet. Several Macintosh computers are also located in the lab and office for image processing, data analysis, literature searchs, sequence analysis, etc. The Silicon Graphics Iris Indigo XS24 graphics workstation at BBRI is available to this project for molecular modeling.

Office: Approximately 100 sq. ft. of office space is allotted to the PI on the second floor of the Boston Biomedical Research Institute.

Other: BBRI has a well equipped machine shop, an in-house library and access to the Treadwell library at the Massachusetts General Hospital. The BBRI has a PerSeptive Biosystems Voyager RP MALDI-TOF mass spectrometer. An in-house fluorescence flow cytometry facility is available. A molecular imager system is available for acquiring and digitizing radioisotopic brain images. There is an X-ray chrystalography facility located at the BBRI. We have access to an in-house Morphology Unit that provides Histology Services, Electron Microscopy Services and Confocal Microscopy Services.

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

Within the laboratory of the PI are: a scintillation counter, pH meter, semi-micro balance, top loading balance, Zeiss inverted tissue culture microscope, CO_2 incubator, fraction collectors, UV monitor, LKB spectrophotometer, microscope, clinical centrifuge, 6 ft. laminar flow hood, 1 liquid N_2 tank (full), flash evaporator, a multiple sample harvester, horizontal DNA/RNA gel electrophoresis apparatus, 2 microfuges, PAGE apparatus, a western blot apparatus, HPLC, and a video fluorescence microscope set-up.

Shared equipment at BBRI includes: a gamma counter, 3 scintillation counters, 2 autoclaves, 4 ultracentrifuges, a peptide sequencer, a warm room, 3 spectrophotometers, cold room, DNA synthesizer, a peptide synthesizer, 3 PCR machines, 4 HPLC units, a flow cytometry facility, EM 300 electron microscope, an AVIV circular dichroism spectrophotometer, a florescence lifetime apparatus, a DU 650 spectrophotometer, a phosphoimager apparatus, a MALDI-TOF mass spectrometer and an ELISA plate reader.

INTRODUCTION: This revision of "Cerebral Delivery of Vectorized Anti-Beta-Amyloid Antibody" addresses the recommendations and constructive criticisms expressed in the IRG summary statement. We have added a new specific aim and methods section to comply with the astute suggestion to infuse our anti-Aß antibodies directly into the brain. Additional preliminary studies have been submitted, including fluorescence micrographs, to demonstrate our ability to prepare transgenic mouse brain sections for Aß plaque analysis. We have also tried to answer each of the poignant questions raised in the summary statement.

(a) Direct Infusion of Anti-Aß into the Brain; The recommendation that we test our anti-Aß antibodies for effectiveness *in vivo* prior to creating bispecific reagents is perfectly logical. Direct infusion provides immediate access to Aß in the brain so that the antibodies do not have to cross the blood-brain barrier. Antibodies that selectively cleave or tightly bind the Aß peptide and/or dissolve its aggregates *in vitro* will be infused into the brain of plaque-bearing Tg2576 transgenic mice. Subsequent action of the antbodies on size and/or number of Aß brain plaques will then be monitored (see Specific Aim A.1c and Research Design and Methods Section D.1c). This valuable preliminary data will help us select anti-Aß antibodies which should produce the best vectorized bispecific reagents for affecting cerebral plaques.

The stereotaxic placement of cannulas needed to perform these infusion studies will initially be performed by surgical technicians at Taconic Technical Services. We have also enlisted the help of Dr. M. J. Young who routinely performs brain cannulation procedures here at our in-house BBRI animal facility. We have and will continue to consult with him regarding these infusion experiments.

While infusion techniques are ideal for demonstrating an immediate effect of our anti-Aß antibodies on plaques, they are not amenable to long-term studies or for the chronic treatment of animals. It should be easier to maintain sustained therapeutic action using systemic bispecific anti-Aß antibodies delivered into the brain via transcytosis.

(b) Properties of the Anti-Aß Antibodies and Bispecfic Reagents; Several questions arose concerning the physical and functional attributes of our antibody-based therapeutic agents. We have attempted to demonstrate in the Preliminary Studies Section that anti-Aß antibodies obtained in our first two hybridoma fusions are not only reactive with Aß but also show catalytic and/or dissaggregative activity *in vitro*. Obtaining the most highly efficient, functionally active anti-Aß antibodies is a continuing aim of this project. That goal will be met as additional hybridoma fusions are performed and more antibodies are screened using increasingly refined assays as noted in (a).

It was correctly pointed out that the Aß substrate within an Alzheimer's disease cerebral plaque is more complex than the Aß aggregates used for our *in vitro* antibody studies. There is some indirect evidence however, that anti-Aß antibodies can react with the Aß of intact plaques (eg. immunohistological staining of brain sections). Irrespective of that possibility, such direct interaction with plaques may not be necessary since ^{125}I-Aß exchange studies on human brain sections indicate that a small amount of soluble Aß exists in equilibrium with plaque-associated Aß. Thus Aß in the plaque should gradually dissociate and become depleted as our antibodies sequester or cleave Aß free in solution.

The cross-linking chemistry needed to produce bispecific antibodies was designed to minimize any possibility of generating new antigenic epitopes. The antibodies were sparingly substituted with SPDP, which yields an innocuous, 4 atom aliphatic linker between the two component halves. Even so, the mice will be monitored for any sign of potential complications due to treatment with our bispecific reagents (see Research Design and Methods Section D.3e). An alternative approach is to crosslink through the hinge thiols which does not require the introduction of exogenous chemicals.

(c) Reversibly of Neuronal Damage Caused by Aß Plaques; Convincing genetic evidence indicates a strong connection between Aß or the plaques it forms and Alzheimer's disease pathology. Thus Aß is a likely target for possible therapeutic intervention. The use of anti-Aß antibodies to

chronically suppress Aß levels in the central nervous system seems to us to be a rational attempt toward preventing or reducing neurological loss.

We agree that impeding the formation of nascent plaques in young transgenic mice should be more successful therapeutically than reducing existing plaques in older mice. However, the model system will allow us to examine both of these important questions. Hopefully, the neuronal damage is not permanent and can be reversed upon dissolution of cerebral plaques.

In the event that neuronal loss persists when Aß plaque formation is prevented then the results would indicate that Aß may not be a primary cause of the disease or its damage is irreversible. In either case, the outcome of our studies should help to determine if there is a direct causal relationship between Aß, the plaques it generates and Alzheimer's disease.

The idea that Aß may down-regulate its own production via a feedback mechanism is an interesting possibilty. As pointed out, if this is the case then using our antibodies to remove Aß would trigger a compensatory output of new Aß. Thus the antibodies might, in effect, do nothing and that would thwart our efforts to treat the disease. Unfortunately, too little is known about the regulation of Aß production to predict if such a feedback mechanism actually exists.

Currently, the main factors influencing Aß generation appear to be α-secretase, β-secretase and γ-secretase along with presenilin-1 which has been shown to be a regulatory cofactor that somehow facilitates γ-secretase activity. Aß is produced from the membrane-bound amyloid precursor protein by β-secretase and γ-secretase cleavage while α-secretase cleavage precludes Aß formation. Aß might down-regulate by inhibiting β or γ either allosterically or via product inhibition, but this type of control is unlikely to occur with proteolytic enzymes. More complex scenarios with Aß binding to a receptor and then regulating presenilin subcellular internalization pathways are also possible, but highly speculative. Ultimately, only further, in-depth experiments will determine if our specifically designed anti-Aß antibodies can effectively deplete cerebral deposits of Aß.

(d) Additional Expertise; The advantages of obtaining expert advice and help from experienced neuroscientists is duly recognized. When we first embarked on this project, guidance and encouragement was obtained from an accomplished neurologist, Dr. L. Recht, who is a long-time friend and collaborator on our early work using immunotoxins to treat brain tumors.

The establishment of a Tg2576 transgenic mouse colony here at BBRI was possible only though the generous cooperation of Dr. Karen Hsiao. In addition to providing breeder mice, she has offered to help solve any problems that may arise regarding these animals (see letter in appendix). In a recent report she has described using a symmetrical Y-maze instead of the Morris water maze for evaluating the behavioral performance of the transgenic mice. We have therefore contacted her to find out if the Y-maze data would be easier for our laboratory to collect and if the results are more straightforward to interpret. If it appears that we are unable to perform these experiments with the help of Dr. Hsiao, we will then actively seek a behavorial collaborator for this aspect of the project.

In order to facilitate the recommended anti-Aß infusion studies we will procure the skilled services of surgical technicians at Taconic. We have and will continue to consult with Dr. Michael Young regarding these newly proposed infusion experiments. He performs brain cannulation procedures for his own research here at our in-house BBRI/SERI animal facility.

We have access to an in-house Morphology Unit that provides histology, electron microscopy and confocal microscopy services. This unit is staffed not only by expert technical people but is overseen by Dr. James Zieske and several principal investigators who are skilled in the latest computer-aided quantitative microscopic techniques. We have direct access to their help and guidance as well as the equipment needed for the analysis of cerebral plaques in the transgenic mice.

A Letter of Support from each of the pertinent scientists mentioned above is included in the Appendix of this application.

(e) New Preliminary Results; It was very exciting to actually visualize the cerebral Aß plaques in mice from our transgenic mouse colony (Preliminary Studies Section C.3a and Fig. 19). Breeder

animals were provided by Dr. Karen Hsiao and we currently have a large number of both young and old (plaque bearing) transgene positive mice which are available for use in the proposed research on intracerebral anti-Aß antibodies.

As described in the Preliminary Studies Section, we began to examine the anti-Aß transition state monoclonal antibodies from our first fusions for catalytic activity. An initial screen for catalytic antibodies in culture medium was performed using a solid phase assay which used a ^{125}I-Aß peptide coupled to a thiol-reactive, iodoacetyl-Sepharose gel. Several clones released soluble ^{125}I-products at a greater rate than other antibodies or the controls. The problem with this assay however is that it did not allow us to determine the identity of the cleaved ^{125}I-peptides or whether the release was truly due to antibody-mediated catalysis.

To obtain more definitive evidence for antibody-mediated cleavage of Aß, we devised a thin layer chromatography-based autoradiography assay (Preliminary Studies Section C.1h and Fig. 12). We also expanded selected anti-Aß transition state clones, induced ascites production and isolated two different monoclonal antibodies using protein A-Sepharose. Peptide cleavage was tested on a Aß 17-mer and the full-length ^{125}I-Aß peptide. Encouraging results were obtained but more experiments must be carried out in this system before we can conclusively state that the antibodies are catalytically hydrolyzing the Aß peptide at the right site.

While the preliminary fusions have yielded some very interesting and potentially catalytic antibodies, additional hybridomas will be produced to optimize our chances for obtaining the best possible antibody-based reagents.

(f) Potential Clinical Relevance; We agree that it is much too early to predict the clinical utility of our immunological approach, especially given that Alzheimer's disease has a very complicated natural history and pathogenesis. There are, of cause, many general aspects about using therapeutic antibodies that are ideally suited to treating human disease. We selected antibodies to target Aß because they are compatible, highly specific molecules that are designed by nature to recognize and seek-out pathogens in the body. Currently, a humanized anti-receptor monoclonal antibody, Herceptin, is giving positive signs in the treatment of breast cancer. The ability of this passively administered antibody to produce a beneficial response in the face of all of the potential physiological hurdles posed by the disease is very promising. Hopefully the fundamental research that we have proposed to perform in an animal model will, in the long run, establish a sound scientific basis for plausibly considering an immunotherapeutic approach to the safe treatment of Alzheimer's disease.

A. SPECIFIC AIMS

Presently, there are few encouraging therapeutic prospects for the prevention or treatment of Alzheimer's disease. However, the reversable deposit of soluble ß-amyloid peptide (Aß) onto plaques in unfixed brain sections and the disintegration of Aß aggregates by monoclonal antibodies suggest that an immunotherapeutic approach to the disease should be feasible. The ultimate objective of this project is to test four central hypotheses which would lay the scientific groundwork for such a therapy. These are: 1) that ß-amyloid plaques in the brain are in equilibrium with soluble Aß peptide; 2) that anti-Aß antibodies can be carried through the blood-brain barrier and into the central nervous system by coupling them to specific vector antibodies; 3) that soluble Aß will be sequestered or depleted by appropriate anti-Aß antibodies in the brain; and 4) that decreasing soluble Aß levels in the central nervous system will either reduce the number or size of preestablished ß-amyloid plaques and/or prevent the *de novo* formation of incipient plaques.

The advantages inherent in the immunotherapy of disease are well established. No harmful drugs or foreign proteins are required for treatment. Immunotherapy is highly specific and can be easily sustained for as long as needed, important features for combating Alzheimer's disease. An ability to experimentally modulate plaque formation in the brain would allow us to unequivocally test the causal relationship between these lesions and memory impairment in affected animals. Our rational assembly of reagents, techniques and model system currently puts us in a unique position to establish the scientific basis for this novel and potentially high impact approach to treating Alzheimer's disease.

The three major research aims are to:

A.1 Elicit Monoclonal Antibodies with Native Aß and Transition State Aß Antigens

a. Immunize mice with several different native Aß and transition state Aß antigens and establish antibody-producing hybridoma clones.

b. Screen and isolate antibodies that dissaggregate Aß or show catalytic activity or have a high binding affinity for the Aß peptide.

c. Test the effects of direct infusion of these antibodies into the central nervous system of Aß plaque-bearing transgenic "Alzheimer's mice."

A.2 Produce Vectorized Anti-Aß/Anti-Receptor Bispecific Antibodies

a. Covalently-link selected anti-Aß antibodies with anti-receptor antibodies to produce bifunctional heterodimers which readily cross the blood-brain barrier.

b. Prepare small bispecific $F(ab')_2$ heterodimers for vector-mediated transport into the brain without the danger of complement fixation.

c. Characterize these bifunctional reagents with respect to their dual specificity and their ability to enter the brain.

A.3 Test Anti-Aß Bispecific Antibodies for Disrupting Plaque Development in Mice

a. Determine if the vectorized bispecific anti-Aß antibodies cross the blood/brain barrier and bind to amyloid plaques found in the brain of "Alzheimer's mice."

b. Treat young "Alzheimer's mice" with vectorized bispecific anti-Aß antibodies and examine the animals for effects on subsequent amyloid plaque development and for the prevention of behavioral deficits.

c. Treat old, plaque-bearing "Alzheimer's mice" with vectorized bispecific anti-Aß antibodies and examine the animals for a reduction in the number or size of preestablished amyloid plaques and reversal of their memory impairment.

41
17
24

B. BACKGROUND and SIGNIFICANCE

B.1 The Importance of the ß-Amyloid Peptide: Alzheimer's disease is a progressive and ultimately fatal form of dementia that affects a substantial portion of the elderly population. Definitive diagnosis at autopsy relies on the presence of neuropathological brain lesions marked by a high density of senile plaques. These extracellular deposits are found in the neo-cortex, hippocampus and amygdala as well as in the walls of the meningeal and cerebral blood vessels. The principal component of these plaques is a 39-43 residue ß-amyloid peptide (Aß). Each plaque contains ~ 20 fmole (80 picograms) of this 4 kDa peptide (1). Apolipoprotein E and neurofibrillary tangles formed by the microtubule-associated tau protein are also often associated with Alzheimer's disease.

Aß is proteolytically cleaved from an integral membrane protein called the ß-amyloid precursor protein. The gene which codes for this protein in humans is found on chromosome 21 (2, 3). Numerous cultured cells and tissues (eg. brain, heart, spleen, kidney and muscle) express this ß-amyloid precursor protein (4) and also secrete the 4 kDa Aß fragment into culture media, apparently as part of a normal processing pathway (5).

While it is difficult to establish an absolute causal relationship between Aß or the plaques it forms and Alzheimer's disease, there is ample evidence to support the pathogenic role of Aß. For example, patients with Down's syndrome have an extra copy of the ß-amyloid precursor protein gene due to trisomy of chromosome 21 (2, 3). They correspondingly develop an early-onset Alzheimer's disease neuropathology at 30-40 years of age. Moreover, early-onset familial Alzheimer's disease can result from mutations in the ß-amyloid precursor protein gene which fall within or adjacent to the Aß sequence (6). These observations are consistent with the notion that deposition of Aß as plaques in the brain might be accelerated by an elevation in its extracellular concentration (7). Of course this mechanism does not exclude other factors from contributing to or causing an increased rate of plaque formation. The finding that Aß is directly neurotoxic both *in vitro* (8) and *in vivo* (9), opens the possibility that soluble aggregated Aß, not the plaques per se, may produce the pathology.

Observations have indicated that amyloid plaque formation may proceed by a crystallization type mechanism (10, 11). According to this model, the seed that initiates plaque nucleation is an Aß which is 42 or 43 amino acids long ($Aß_{1-43}$). The rate-determining nucleus formed by $Aß_{1-43}$ or $Aß_{1-42}$ allows peptides $Aß_{1-40}$ or shorter to contribute to the rapid growth of an amyloid deposit. This nucleation phenomenon was demonstrated *in vitro* by the ability of $Aß_{1-42}$ to cause the instantaneous aggregation of a kinetically stable, supersaturated solution of $Aß_{1-40}$. That finding has led to the possibility that $Aß_{1-40}$ might be relatively harmless in the absence of the nucleation peptides $Aß_{1-42}$ or $Aß_{1-43}$. Indeed, elevated levels of the long peptides were found in the blood of patients with familial Alzheimer's disease (7). Moreover, $Aß_{1-42}$ or $Aß_{1-43}$ was the overwhelmingly predominant form deposited in the brain plaques of many Alzheimer's disease patients (12).

One might expect that Aß plaque formation in the brain is an irreversible process with little chance for therapeutic intervention. However, important experiments suggest that a dynamic equilibrium may exist between soluble Aß and fibrillar Aß deposited as plaques in the brain. Exogenous, soluble ^{125}I-$Aß_{1-40}$ was shown by autoradiography to specifically deposit into the plaques of unfixed brain tissue sections obtained from postmortem Alzheimer's disease patients (13). Normal brain had no binding sites and the association of ^{125}I-$Aß_{1-40}$ with tissue from Alzheimer's disease patients was displaced by unlabeled Aß. Moreover, once incorporated into the plaque, this ^{125}I-$Aß_{1-40}$ slowly dissociated with a half-time of ~ 1h. The fact that ^{125}I-$Aß_{1-40}$ peptide bound specifically, reversibly and with high affinity to brain plaques suggests that a steady-state equilibrium exists between Aß in plaques and Aß free in solution (13-15).

Similar conclusions can be drawn from experiments performed using an entirely different approach. Harsh detergents or strong acids are required to dissolve amyloid plaques and artificial Aß aggregates formed *in vitro*. However, it has recently been demonstrated that mild treatment with certain anti-Aß monoclonal antibodies can effectively dissolve preformed Aß aggregates (16). Since these antibodies also inhibit the initial aggregation of the peptide (17), the results indicate that self-aggregates ($Aß_n$) exist in equilibrium with a very small amount of monomer (nAß).

$$Aß_n(\text{agg.}) \leftrightarrow nAß$$

When an appropriate antibody (Ab) is added to this system it apparently binds the monomer and dissolves the aggregate by pulling the reaction to the right.

$$A\beta_n(\text{agg.}) \rightarrow nA\beta + Ab \rightarrow nA\beta{:}Ab$$

These findings are important because they support the idea that plaques exist in an equilibrium state with soluble monomer. They also provide hope for using antibodies or their small fragments as a gentle means for eradicating plaques in Alzheimer's patients.

B.2 Reduction of ß-Amyloid Peptide Pools in the Brain: The preceding observations speak to the central hypothesis of this proposal, namely that the onset of plaque formation, plaque size or the number of plaques should be influenced by reducing levels of free Aß in the brain. This will be accomplished by using anti-Aß antibodies selected to either tightly bind Aß, or solublize the aggregated Aß present in plaques, or to inactivate the peptide by catalytic cleavage (18-21). Thus, the antibodies will act as an Aß sink which prevents the deposit of new soluble Aß onto existing plaques and/or dissolves preestablished plaques in the brain by pulling the equilibrium towards soluble Aß.

It is well established, however, that induced endogenous antibodies or antibodies administered passively into the peripheral circulation do not normally cross the blood-brain barrier. Blood-borne anti-Aß antibodies therefore, cannot reach Aß plaques or soluble Aß in the central nervous system. Fortunately, a universal method for the rapid, vector-mediated delivery of macromolecules across the blood-brain barrier has been devised (22-25). This novel system can be adapted to carry anti-Aß antibodies over the blood-brain barrier so that they are in immediate contact with the Aß plaques and soluble Aß pool in the brain (Fig. 1).

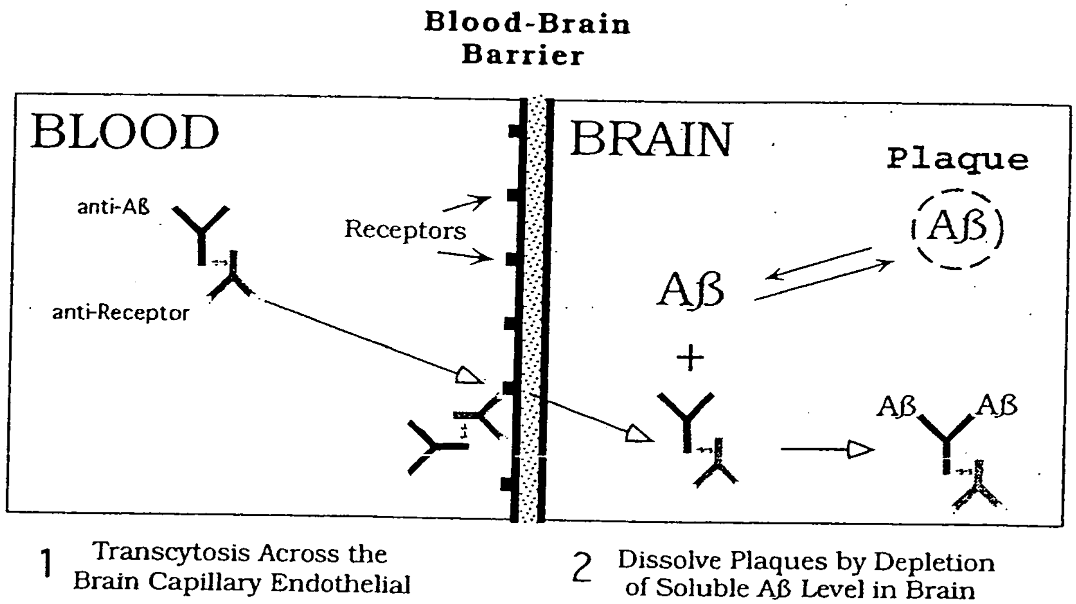


Fig. 1 Vector-Mediated Delivery of Anti-Aß into the Brain

A vector moiety must be chemically or genetically attached to the anti-Aß antibody to facilitate its delivery into the central nervous system. This vector component could be, for example, an anti-transferrin receptor or anti-insulin receptor antibody that binds to those receptors on the brain capillary endothelial cells (22-25) which make up the blood-brain barrier. The resulting bifunctional antibody (26-30) will attach to appropriate receptors on the luminal side of the vessel (Fig. 1). Once bound to the receptor, both components of the bispecific antibody can pass across the blood-brain barrier by the process of transcytosis. Anti-Aß antibodies which have entered the brain will interact directly with both Aß plaques and the soluble Aß pool. It has been estimated that concentrations of macromolecules in the 10^{-8}-10^{-7}M range can be achieved in the brain using vector-mediated delivery via these brain capillary enriched protein target sites (22,29). Importantly, the vector appears safe since animals dosed daily for two weeks with an anti-transferrin receptor antibody showed no loss of integrity of the blood-brain barrier using a radioactive sucrose probe (31).

Depending on their design, anti-Aβ bispecific antibodies situated in the brain can function in three different ways to reduce soluble Aβ and Aβ deposits. An anti-Aβ bispecific antibody that tightly binds soluble Aβ will not only sequester the peptide but, due to efflux of vectorized molecules from the central nervous system (32), potentially can carry the bound Aβ out of the brain and release it into the blood stream. This clearance mechanism would lead to a continuous cycling of Aβ out of the brain (Fig. 2, path 1). The use of site-directed anti-Aβ antibodies which can directly dissolve Aβ aggregates (16) adds a new dimension to our strategy. Antibody-mediated dissaggregation of fibrilar Aβ rendered it nontoxic *in vitro*. Importantly, a low ratio of antibody to Aβ (1:10) was effective for this conversion (16) so that a similar effect could be achieved on the Aβ plaques in the brain (Fig. 2, path 2). Another attractive possibility that we will explore makes use of catalytic anti-Aβ antibodies (18-21) designed to cleave Aβ into harmless fragments. The advantage of delivering an Aβ-specific catalytic antibody into the brain is two-fold. The Aβ peptide would be permanently destroyed by such antibodies and, since catalysis is continuous, each antibody will inactivate many target Aβ molecules in the brain (Fig. 2, path 3). Thus much less vectorized bispecific antibody would have to be delivered into the central nervous system to achieve the desired depletion of Aβ.

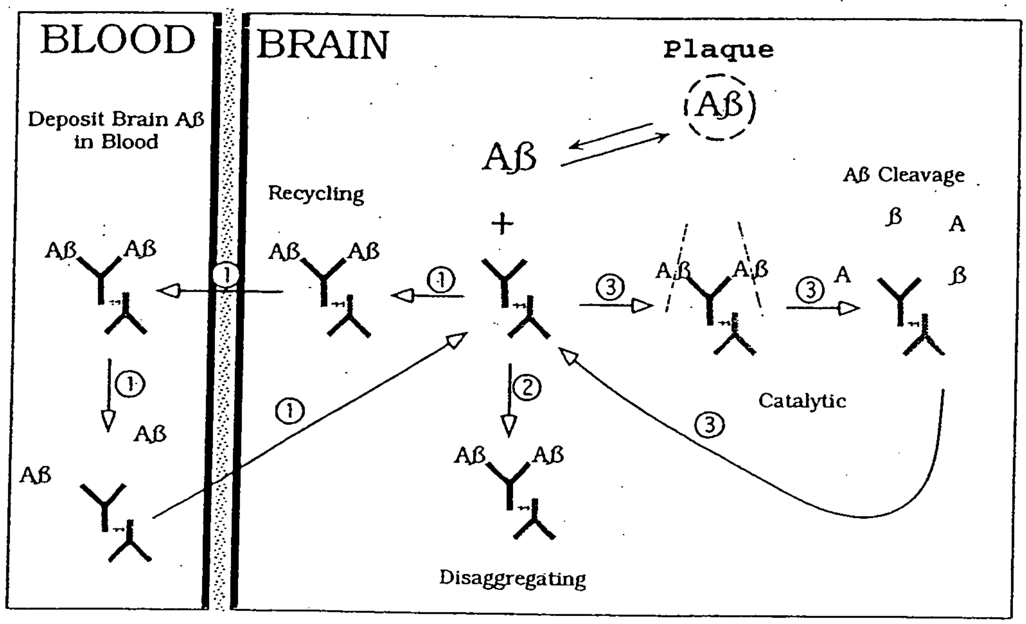


Fig. 2 Three Modes of Anti-Aβ-Mediated Depletion of Aβ from the Brain

To be effective the anti-Aβ sites of the bispecific antibody must be empty before passage out of the blood and into the brain (Fig. 1). Therefore the concentrations of bispecific antibody that are sustainable in animals must exceed the level of Aβ circulating in the blood. In both controls and patients with sporadic Alzheimer's disease, plasma concentrations of $Aβ_{1-40}$ and $Aβ_{1-43}$ were 200 pM and 30 pM, respectively while patients with familial Alzheimer's disease had only 2-3-fold higher levels, 600 pM and 60 pM, respectively (7). The medium-range plasma level of bispecific antibody expected in a treated animal is ~150 μg/ml or 1,000,000 pM. Since this greatly surpasses plasma Aβ, 99.9% of the bispecific antibodies that enter the brain will have unoccupied anti-Aβ combining sites. Moreover, the use of catalytic anti-Aβ antibodies that continuously destroy Aβ, would provide a highly efficient and permanent means of depleting these peptides in both the blood and the brain.

B.3 Application of Catalytic Antibodies to Alzheimer's Disease: More than 20 years ago, Raso and Stollar published the first formal study expressly aimed at inducing antibodies possessing catalytic activity (18-20). A transition state enzyme inhibitor was designed, synthesized and used as a hapten to elicit complementary antibody combining sites that would mimic the chosen enzyme active site. A fivefold rate enhancement was achieved for the tyrosine transamination reaction occurring at these antibody sites versus free in solution. This modest acceleration is actually quite significant, considering that this result was obtained well before development of hybridoma technology, so only heterogeneous populations of affinity-purified rabbit serum antibodies could be used.

With the emergence of monoclonal antibody techniques, the field of catalytic antibodies has exploded, largely due to recent efforts from the laboratories of Lerner, Benkovic and Schultz (21). Homogeneous catalytic antibodies can now be selected, purified and studied in the absence of any competing non-catalytic species. Numerous catalytic antibodies, accelerating a large array of diverse chemical reactions, have been produced within the last several years (21, 33-38). In light of the rapid progress since our early pioneering work, it is apparent that the time is now ripe to apply this unique technology to the pressing health problems confronting medical scientists.

With this end in mind, we have begun to design antibodies that will specifically cleave Aß which forms the amyloid plaques in the brain of Alzheimer's patients. Studies have indicated that the deposit of $Aß_{1-43}$ in senile plaques (12) is central to Alzheimer's disease pathogenesis and shortened versions of the ß-amyloid peptide may be less of a problem in the absense of $Aß_{1-43}$ (10, 11). Given the importance of $Aß_{1-43}$ to plaque formation, any premature, illegitimate or preemptive cleavage of this peptide by a catalytic antibody should yield an amyloidogenically inactive and less harmful form of Aß. Each antibody would permanently inactivate many target molecules rather than just acting stoichiometrically. Thus the progression of Alzheimer's disease would be impeded by attenuating or abolishing the plaque forming capability of Aß in the brain. Transition state antigens and monoclonal antibodies produced in this project could be useful in combating Alzheimer's disease by providing a means to specifically destroy $Aß_{1-43}$.

An advantage to choosing Aß as a target for developing catalytic antibodies is that carrier-bound, small Aß peptides readily elicit specific monoclonal antibodies (39-43). Antibody binding to Aß can occur when Aß is incorporated into senile plaques. A large array of Aß peptide antigens has been used to generate antibodies (39-43). Importantly, antibodies which can distinguish $Aß_{1-42}$ and $Aß_{1-43}$ from $Aß_{1-40}$ have been raised using the carboxy-terminal sequence (39).

B.4 Transgenic Mouse Model of Alzheimer's Disease: The effect of catalytic or conventional antibodies on Aß equilibria will be studied in a transgenic mouse model of Alzheimer's disease (44). This strain carries a gene for the human amyloid precursor protein containing the Swedish family early-onset Alzheimer's disease double mutation $Lys_{670} \rightarrow Asn$ and $Met_{671} \rightarrow Leu$. Inserted into a hamster prion protein cosmid vector, this transgene produced the highest levels of human amyloid precursor protein in brain and spinal cord but other tissues including muscle, skin, lung and heart also expressed the gene product (45). A 5-fold increase of $Aß_{1-40}$ and a 14-fold increase in $Aß_{1-43}$ was found in the brain of ~1 year old mice while younger animals showed little or no increase. This age-related elevation in Aß levels correlated with the appearance of brain plaques and memory deficits (44), thus closely mimicking the manifestations of Alzheimer's disease in humans.

B.5 Summary: The proposed study will focus on only one aspect of Alzheimer's disease which has a very complicated natural history and pathogenesis. This novel immunological approach however, is predicated on the likelihood that altering the Aß levels in the brain will ameliorate the course of Alzheimer's disease, at least temporarily. This research will identify and characterize peptide antigens that elicit anti-Aß antibodies which selectively cleave, or tightly bind the Aß peptide and/or dissolve its aggregates. Those antibodies will be linked to an anti-receptor antibody which will serve as a vector for delivery across the blood-brain barrier and into the brain. The anti-Aß bispecific antibodies are designed to displace Aß equilibria by permanently inactivating Aß, or by specifically sequestering the peptide to directly disaggregate plaques and/or redistribute soluble brain Aß to the peripheral circulation. Our hypothesis suggests that the specific anti-Aß antibodies will act as a sink which, by lowering the level of soluble Aß in the brain, should reduce or prevent plaque formation.

We will test these anti-Aß bispecific antibodies for perturbing Aß equilibria in a transgenic mouse model of Alzheimer's disease. This model system will also allow us to evaluate the functional consequences of depressed Aß levels in the brain on the course of the disease. Reversing or preventing the symptoms of disease in these mice with anti-Aß bispecific antibodies would help to establish a causal relationship between Aß, the plaques it forms and Alzheimer's disease. This is one of the gaps in knowledge that the proposed research is designed to fill. We will proceed to refine the bispecific antibodies to achieve a longer term objective, the production of clinically useful immunoreagents for the prolonged and safe treatment or prevention of Alzheimer's disease.

C. PRELIMINARY STUDIES

C.1 Elicit Monoclonal Antibodies with Native Aß and Transition State Aß Antigens

a. Synthesis of ß-amyloid peptide antigens: The amino acid sequence of the 43 residue ß-amyloid peptide (Aß) is shown in Fig. 3. Predicting precisely which site on the Aß peptide will be ultimately best suited for antibody-mediated therapy is difficult. Therefore 3 key epitopes on the

H_2N-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIAT-CO_2H

1 10 20 30 40

Fig. 3

Aß 43-mer were chosen as targets for the catalytic and conventional antibodies. The resulting panel of monoclonal antibodies will be screened *in vitro* to identify desirable properties such as high affinity binding, catalytic activity and/or the ability to dissociate Aß aggregates. Select anti-Aß antibodies will then be vectorized by coupling to an anti-transferrin receptor antibody (anti-TfR) and studied in the Tg mouse model to determine empirically which of these unique immuno-reagents are both therapeutically effective and pharmacologically safe.

Peptide antigens for elicting an immune response directed against the amino-terminus, the central region and the carboxy-terminus of Aß were produced (Fig. 4). A Cys residue was added and the peptides were synthesized using standard automated Fmoc chemistry. The peptides were purified by HPLC and their composition was verified by mass spectral and amino acid analysis. The Cys substitution was designed to provide a sulfhydryl linkage group for coupling the peptides to antigenic, maleimide-activated carrier proteins such as Keyhole Limpet Hemocyanin (KLH).

H_2N-Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Cys-amide

1 5 10 15

Cys-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-amide

10 15 20 25

Cys-Met-Val-Gly-Gly-Val-Val-Ile-Ala-Thr-CO_2H

35 40

Fig. 4

b. Phosphonamidate and phosphonate based transition state peptides: A phosphonamidate transition state analog encompassing the carboxy-terminal region of Aß has been synthesized (Fig. 5).

↓

N-acetyl-Cys-Met-Val-Gly-Gly--**CO-NH**--Val-Val-Ile-Ala-amide

35 40

N-acetyl-Cys-Met-Val-Gly-Gly-**PO_2^--NH-**Val-Val-Ile-Ala-amide

Fig. 5

Replacement of the proposed scissile peptide linkage between Gly_{38} and Val_{39} (↓) with a phosphonamidate moiety (**-PO_2^--NH-**) is designed to elicit catalytic antibodies that will hydrolytically cleave Aß at this site. The N-acetyl-Cys residue was placed at the position of Leu_{34} to provide

a suitable linkage group for coupling this peptide to an antigenic carrier protein. The structures shown in Fig. 6 represent the putative transition state for peptide hydrolysis by zinc peptidases and the phosphonate and phosphonamidate mimics. Similar tetrahedral transition state

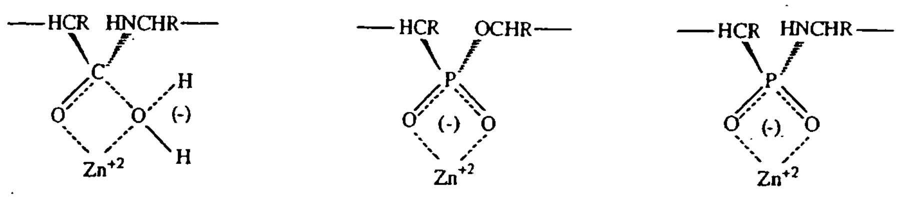


Fig. 6 Transition-state Phosphonate Phosphonamidate

intermediates are formed in each of the four classes of proteolytic enzymes, the serine-, cysteine-, aspartic- and metallo-peptidases.

The design strategy and methods for synthesizing phosphonamidate based transition state peptides are straightforward (46, 47). The N-terminal portion of the peptide (N-acetyl-Cys-Met-Val-Gly) was made using standard automated Fmoc chemistry. Its amino terminus was capped with acetic anhydride while it was still attached to the resin. After cleavage from the resin the N-acetyl tetrapeptide was treated with pyridine disulfide to protect its sulfhydryl group. An acid chloride of Cbz-glycine phosphonate monomethyl ester (46, 47) was coupled with Val-Val-Ile-Ala-amide which was synthesized by automated Fmoc chemistry. The last amino acid of Aß, Thr, was omitted because of potential problems with its unprotected hydroxyl group. The product, Cbz-Gly-PO_2^--NH-Val-Val-Ile-Ala-amide has a phosphonamidate (methyl ester) bond between the Gly and Val residues. Next, the Cbz blocking group was removed using hydrogen so that the protected N-acetyl-Cys-Met-Val-Gly peptide could be added to the amino terminal end of this transition state peptide by HBTU-activated peptide linkage. Treatment with mercaptoethanol and rabbit liver esterase was used to deblock the peptide. Each key component listed in the synthetic scheme was tested for purity by HPLC and its composition was verified by mass spectral and amino acid analysis. This new Aß analog, N-acetyl-Cys-Met-Val-Gly-Gly-**PO_2^--NH**-Val-Val-Ile-Ala-amide (Fig. 5) is designed to elicit catalytic antibodies that will specifically cleave Aß at the Gly-Val bond.

The synthesis of phosphonate Aß transition state peptides (eg. N-acetyl-Cys-Met-Val-Gly-Gly-**PO_2^--O**-Val-Val-Ile-Ala-amide) will follow a similar scheme and will use some of the same intermediates described for the phosphonamidate transition state analog.

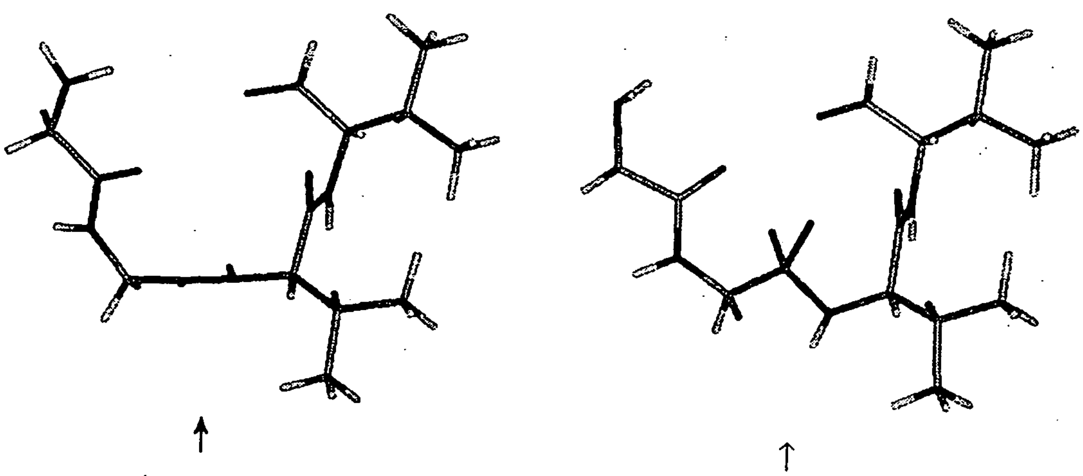


Fig. 7 Gly-Gly-**CO-NH**-Val-Val Gly-Gly-**PO_2^--NH**-Val-Val

A structural comparison was made between the native Aß peptide and the transition state phosphonamidate Aß peptide (Fig. 7) using a graphics workstation. The peptide link **-CO-NH-** (↑) between Gly_{38} and Val_{39} was replaced with a phosphonamidate bond **-PO_2^--NH-** (↑) and an energy minimization was applied. The orientation shown above, Fig. 7, clearly illustrates the difference

between the planar peptide link **-CO-NH-** (↑) of native Aß on the left versus the corresponding tetrahedral phosphonamidate bond **-PO_2^--NH-** (↑) in the transition state peptide on the right.

An antibody combining site complementary to the tetrahedral transition state analog on the right of Fig. 7, will force the normally planar bond of the Aß substrate peptide on the left into a transition state-like conformation. Such bond distortion can catalyze the hydrolytic cleavage of the Aß peptide at the Gly_{38}-Val_{39} linkage.

c. Statine based transition state peptides: A series of statine **(Sta)** transition state analogs encompassing the carboxy-terminal region of Aß (Cys-Met-Val-Gly-Gly-Val/**Sta**-Val/**Sta**-Ile/**Sta**-Ala-Thr) has been synthesized in this laboratory (Fig. 8).

↓ ↓ ↓

Met-Val-Gly-Gly-Val-Val-Ile-Ala-Thr-CO_2H

35 40

Fig. 8

Replacement of the proposed scissile peptide linkage between Val_{39} and Val_{40} (↓), Val_{40} and Ile_{41} (↓) and Ile_{41} and Ala_{42} (↓) with a "statyl" moiety (-CHOH-CH_2-CO-NH-) is designed to elicit catalytic antibodies that will hydrolytically cleave Aß at one of these sites. A Cys residue was placed at the position of Leu_{34} (Fig. 3) to provide a suitable linkage group for coupling this peptide to a maleimide-activated carrier protein.

The statine transition state peptides were entirely synthesized using standard automated Fmoc chemisty. This was feasible due to the availability of Fmoc-statine **(Sta)**, [N-Fmoc-(3S,4S)-4-amino-3-hydroxy-6-methyl heptanoic acid] from a commercial source. Each peptide was tested for purity by HPLC and its composition was verified by mass spectral and amino acid analysis.

d. Phenylalanine statine based transition state peptides: A series of phenylalanine statine **(PhSta)** transition state analogs encompassing the central region of Aß (Cys-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe/**PhSta**-Phe/**PhSta**-Ala-Glu-Asp-Val-Gly-amide) was synthesized in this laboratory (Fig. 9).

↓ ↓

Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-amide

10 15 20 25

Fig. 9

Replacement of the proposed scissile peptide linkage between Phe_{19} and Phe_{20} (↓) and Phe_{20} and Ala_{21}(↓) with a statyl moiety (**-CHOH-CH_2-CO-NH-**) (Fig. 9) is designed to elicit catalytic antibodies that will hydrolytically cleave Aß at these sites (Fig. 9). A Cys residue was placed at the position of Gly_9 (Fig. 3) to provide a sulfhydryl linkage group for coupling the peptide to antigenic, maleimide-activated carrier proteins such as KLH.

The phenylalanine statine transition state peptides were entirely synthesized using standard automated Fmoc chemisty. This was feasible due to the recent availability of Fmoc-"phenylalanine statine" **(PhSta)**, [N-Fmoc-(3S,4S)-4-amino-3-hydroxy-5-phenylpentanoic acid] from a commercial source. Each peptide was tested for purity by HPLC and its composition was verified by mass spectral and amino acid analysis.

An antibody combining site that is complementary to the elongated tetrahedral transition state analog will force the normally planar peptide bond of the Aß substrate into a transition state-like conformation. Such distortion should catalyze the cleavage of Aß at the Phe_{19}-Phe_{20} bond.

e. Immunization of mice: Standard protocols were used to immunize BALB/c mice with the KLH-linked Aß peptides described in the preceding sections. Briefly this procedure used i.p. injection of the different antigens emulsified in complete Freunds adjuvant, followed by a second course in incomplete Freunds adjuvant. Three days prior to the hybridoma fusion, the mice were boosted i.v. with antigen in PBS.

f. ^{125}I-Aß binding assay: It was very important to demonstrate that our anti-Aß and anti-transition state Aß monoclonal antibodies bind to the natural $Aß_{1-43}$ peptide which they are designed to cleave. Therefore we radiolabeled $Aß_{1-40}$ and $Aß_{1-43}$ with ^{125}I and then separated the iodinated peptide from unlabeled material by HPLC to give essentially quantitative specific activity (~2000 Ci/mmol) (13). This probe was incubated for 1h at 23°C with either purified anti-Aß antibodies or media taken from hybridoma clones producing anti-Aß antibodies. A polyethylene glycol separation method was used to detect the amount of ^{125}I-$Aß_{1-43}$ bound to antibody (Table I).

Table I **^{125}I-$Aß_{1-40}$ Binding to a Purified Monoclonal Anti-Aß Antibody ***

Testing specific binding activity

Addition	^{125}I-$Aß_{1-40}$ Bound (cpm)	Specifically Bound (% of total added)
Control	8,560	-
+ 5A11 anti-Aß	64,589	79

* anti-Aß 5A11 at 2x10^{-6} M; Added ~70,000 cpm of ^{125}I-$Aß_{1-40}$

The data in Table I demonstrate the ability of our purified 5A11 monoclonal anti-Aß antibody to bind a high percent of ^{125}I-$Aß_{1-40}$. This binding assay will be valuable to screen clones and purified antibodies (Table I) for their ability to bind Aß and can also serve as the basis for a competitive displacement assay to measure the relative binding strength of different unlabeled Aß peptides. With very efficient catalytic antibodies this binding assay may have to be performed on ice to ensure that no cleavage of Aß occurs during the 1h incubation time. The assay will allow us to quickly identify clones which produce high affinity anti-Aß antibodies.

g. Hybridoma production I: We performed a hybridoma fusion using the spleen of a mouse immunized with the phenylalanine statine transition state Aß-KLH antigen (Fig. 9). Monoclonal antibodies from several of the hundreds of hybridoma supernatants produced were screened using ELISA to assess their binding to both the normal $Aß_{1-43}$ peptide and to the phenylalanine statine transition state Aß peptide. Two major patterns were found (Fig. 10).

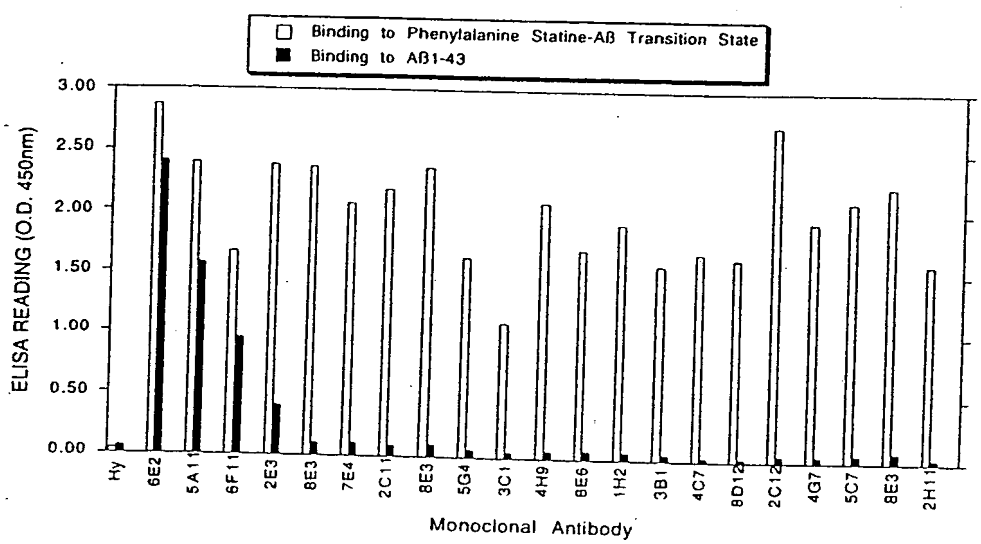


Fig. 10 ELISA Comparing Antibody Binding to Transition State Versus Native Aß

One group of antibodies (at the left of Fig. 10) bound to the immunizing transition state peptide and cross-reacted strongly with the native $A\beta_{1-43}$ peptide when each was adsorbed directly onto the ELISA plate. A second group (at the right) showed a high binding preference for the phenylalanine statine transition state Aβ peptide and reacted minimally with native $A\beta_{1-43}$.

Strong color reactions were obtained in this ELISA using only 10 μl of hybridoma supernatent while Hy media alone or PBS gave a low background (Fig. 10). These results demonstrate that the comparative ELISA screen, although only a semi-quantitative measure of binding, will provide a means for choosing monoclonal antibodies that are highly selective for, and most reactive with, the transition state. Importantly, the antibodies bound to the carrier-free Aβ peptides adsorbed directly onto microtitre plates, showing their anti-peptide specificity.

These findings indicate that several of the new anti-Aβ transition state antibodies are unique. They can bind to both the phenylalanine statine- and normal-Aβ peptides. Their selective recognition of the transition state and weaker cross-reaction with native $A\beta_{1-43}$ however implies that this binding interaction is very different from that shown by conventional anti-native Aβ antibodies. It suggests further that these new antibodies may be able to force the native Aβ peptide into a conformation resembling the transition state for hydrolytic cleavage. Importantly, some of the antibodies which showed only minimal binding to $A\beta_{1-43}$ in this ELISA, did display cross-reactivity with the natural peptide using a highly sensitive ^{125}I-$A\beta_{1-43}$ binding assay (Table I).

ssay
roteolytic
ctivity

h. Solid phase and TLC Aβ proteolytic assays: A solid phase ^{125}I-labeled Aβ assay was developed to screen anti-transition state antibody hybridoma supernatants for specific proteolytic activity. The Cys-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Tyr-amide peptide encompassing amino acids 14-25 of Aβ (Fig. 3) was synthesized with a Cys and Tyr added at either end. This was radiolabeled with ^{125}I and the iodinated peptide was then separated from unlabeled material by HPLC to give essentially quantitative specific activity (~2000 Ci/mmol). The highly radioactive Aβ peptide was coupled to a thiol-reactive, iodoacetyl-Sepharose gel to form an irreversible linkage. Catalytic antibodies should promote the progressive release of soluble ^{125}I-peptide from the solid phase matrix. The proposed assay was verified by the ability of several different proteases in to rapidly hydrolyze this Sepharose-linked Aβ substrate. The peptide is readily accessible to proteolytic cleavage as revealed by the release of soluble ^{125}I-peptide that increased with incubation time (data not shown). Selected antibodies were screened for catalytic activity using release of radioactivity from ^{125}I-Aβ-Sepharose (Fig. 11).

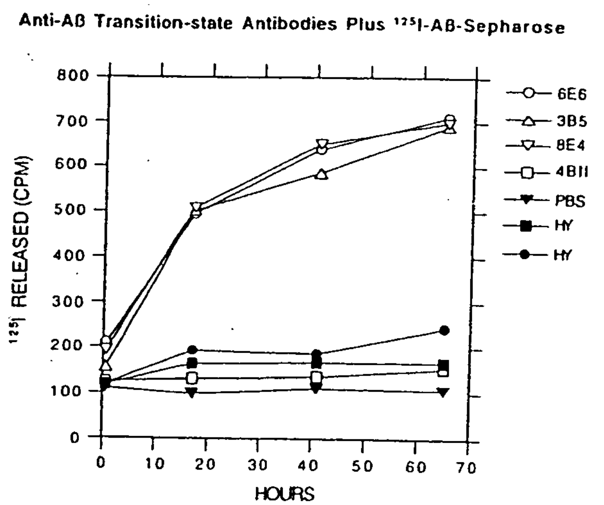


Fig. 11 Cleavage of ^{125}I-Aβ-Sepharose

The results obtained at pH 7, 25°C indicate that the antibody-containing media of several clones released ^{125}I-peptide at a greater rate than other clones from this fusion or the PBS and Hy medium controls (Fig. 11). Large amounts of these antibodies will now be obtained, purified and tested a higher concentrations to achieve much faster rates of cleavage and to verify that the antibodies are acting in a catalytic mode using conventional enzyme kinetics. By changing the composition of the ^{125}I-peptide we can use this same strategy assay antibodies reactive with different regions of Aβ.

We devised a thin layer chromatography-based autoradiography assay so that more definitive evidence for antibody-mediated cleavage of Aβ could be obtained. We also expanded selected anti-phenylalanine statine Aβ transition state clones, induced ascites production and isolated the different monoclonal antibodies using protein A-Sepharose. The cleavage assay used ^{125}I-$A\beta_{1-40}$ and a 17-mer, encompassing amino acids 9-25 (Fig 3). These two ^{125}I-labeled peptides bound to the purified monoclonal antibodies 5A11 and 6E2 when examined using either a PEG precipitation assay or by a co-electrophoresis method. To test for peptide cleavage we added the antibodies to the ^{125}I-peptides, allowed them to incubate and then spotted the reaction mix onto polyamide thin layer

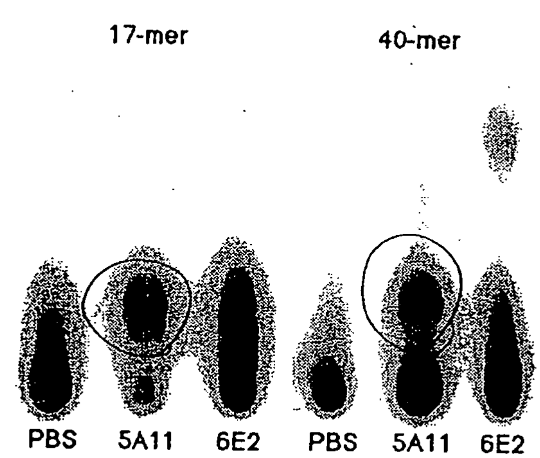


Fig. 12 TLC of ^{125}I-Aβ-Cleavage

Separating cleaved fragments

sheets. The chromatographs were developed in different solvents (eg. 0.5N HCl, 0.5N NaOH or pH7 phosphate buffer) and the migration of ^{125}I-products was followed by exposing the sheet using a quantitative phosphoimager system (Fig. 12).

It is very encouraging to see that these antibodies break down the Aβ peptides compared to the untreated peptides (PBS). Obviously, many more experiments must be performed and additional controls will have to be run before we can conclusively state that the antibodies are catalytically hydrolyzing the Aβ peptide at the right site. Various naturally occurring proteases will be tested in this system so that we can identify the cleavage site of the antibodies by comparison with the known specificity of the different enzymes. We will also sequence the cleaved Aβ peptides.

i. Hybridoma production II: Another distinct hybridoma fusion was performed using the spleen of a mouse immunized with a KLH conjugate of the statine (**Sta**) transition state analogs encompassing the carboxy-terminal region of Aβ (Fig. 8) (Cys-Met-Val-Gly-Gly-Val/**Sta**-Val/**Sta**-Ile/**Sta**-Ala-Thr). ELISA was used to demonstrate antibody binding to both the normal Aβ$_{1-43}$ peptide and to the statine transition state Aβ peptide (Fig. 13).

The antibodies bound to the C-terminal locus on these carrier-free Aβ peptides adsorbed directly to the microtitre plate, confirming their anti-peptide specificity. Most of the antibodies preferentially recognized the statine Aβ transition state but cross-reacted with native Aβ$_{1-43}$. This suggests that these new antibodies may be able to force the native Aβ peptide into a conformation resembling the transition state for hydrolytic cleavage of its C-terminal amino acids. Such cleavage would in effect convert Aβ$_{1-43}$ into potentially less harmful shorter peptides, like Aβ$_{1-40}$ or Aβ$_{1-39}$.

Clone 11E9 had the strongest binding preference for the statine analog and might therefore be the best prospect for having catalytic activity (Fig. 13). Several clones displayed no difference in their reactivity with the native versus statine transition state Aβ peptide. We also tested the clones with Aβ$_{1-40}$ to identify antibodies which do not react with this shortened, 40 amino acid version of Aβ (Fig. 13). Used therapeutically, such antibodies would preferentially bind/cleave the less abundant but more noxious Aβ$_{1-43}$ species in the blood as opposed to the smaller and less detrimental Aβ$_{1-40}$.

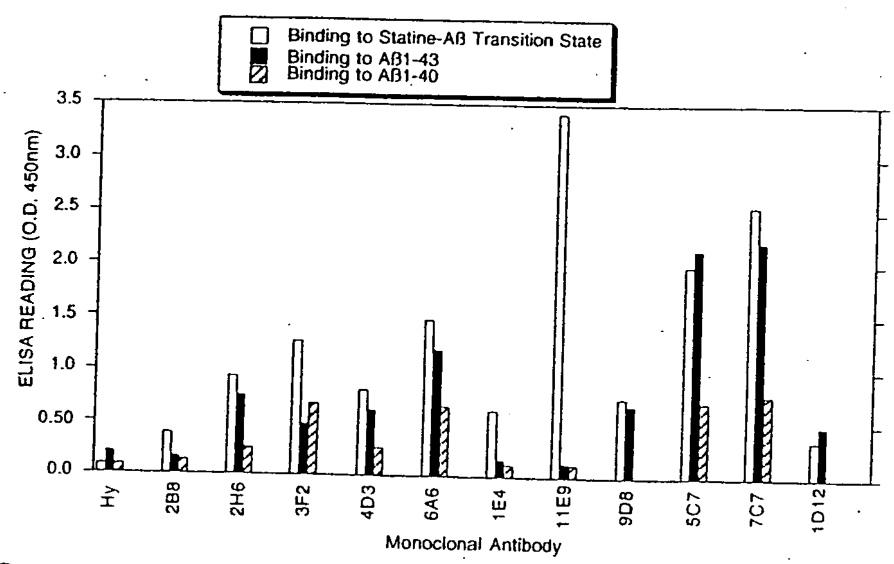
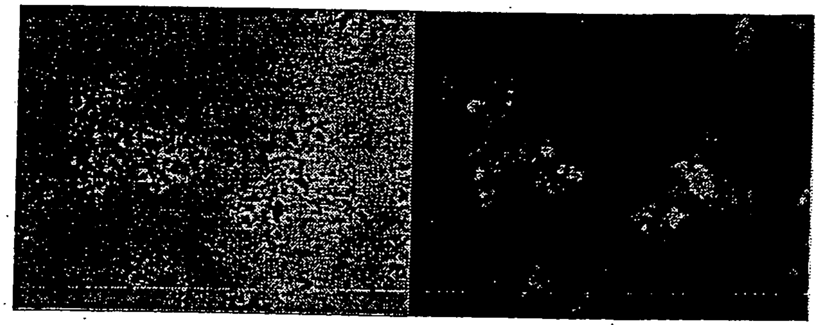


Fig. 13 ELISA Comparing Antibody Binding to Transition State Versus Native Aβ

j. Disaggregation of ß-Amyloid by Monoclonal Antibodies: The self-aggregation of synthetic Aß peptides leads to microscopic structures which resemble amyloid plaques in the brain (16, 17) and exhibit the same bright green fluorescence upon exposure to thioflavin T (Fig. 14). These aggregates are very stable and usually require harsh detergents or strong acids to dissolve. However, it has now been demonstrated that the binding of certain anti-Aß monoclonal antibodies can effectively inhibit the initial aggregation of this peptide and also disaggregate preformed Aß complexes (16, 17).



Phase Contrast Thioflavin Fluorescence

Fig. 14 Photomicrographs of Self-Aggregated Aß Peptide

We set up a radioactive assay as a quick way to screen the different monoclonal antibodies produced in this laboratory for an ability to dissolve preformed Aß aggregates. After adding ^{125}I-Aß to unlabeled soluble peptide, aggregates were formed by bringing the solution to pH 5 or by stirring it overnight in PBS. An aliquot of the labeled aggregate was incubated for 1hr with either PBS, the 5A11 anti-Aß antibody or an equal amount of an irrelevant mouse antibody (7D3, anti-human transferrin receptor). After centrifugation, the level of radioactivity in the precipitate was measured (Table II). The fact that the Aß-specific 5A11 antibody solubilized 80% of the Aß aggregates while an equal amount of the control antibody had only a minor effect suggests that the equilibrium was displaced by antibody-mediated binding of soluble Aß.

Table II Solubilization of ^{125}I-Aß$_{1\text{-}40}$ Aggregate by Monoclonal Anti-Aß Antibody

Addition	^{125}I-Aß$_{1\text{-}40}$ in Ppt. (cpm)	Amount Solubilized (% of PBS Control)
PBS control	3,420	-
+ 5A11 anti-Aß	676	80
+ 7D3 anti-TfR	2,458	27

C.2 Produce Vectorized Anti-Aß/Anti-Receptor Bispecific Antibodies

a. Vectors for transcytosis across the blood-brain barrier: Anti-transferrin receptor antibodies (anti-TfR) are the primary vectors that we will deploy for delivery of the anti-Aß antibodies into the brain. The 7D3 mouse monoclonal antibody developed in this lab, is specific for the human receptor and selectively immunostains cortical capillaries in normal human brain tissue (48) (Fig. 15).

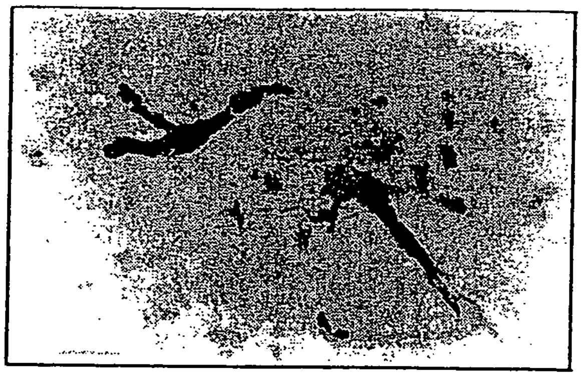


Fig. 15 Human Brain Capillaries Stained with the 7D3 Anti-TfR (48)

Antibody attachment to the receptor is not blocked by an excess of human transferrin. The epitope recognized by this antibody is therefore distant from the receptor-ligand binding site. Bispecific antibodies constructed with this 7D3 antibody and an anti-Aß antibody would be potentially useful for therapy in patients with Alzheimer's disease and possibly for preclinical trials in primates.

For studies in the transgenic mouse model of Alzheimer's disease we have obtained an anti-mouse transferrin receptor monoclonal antibody produced in the rat. This antibody also appears to recognize a transferrin receptor epitope which does not involve ligand binding. The antibody therefore has no effect on cell proliferation when tested using murine lines.

b. Synthesis of bispecific antibodies: The anti-Aß antibodies have been chemically coupled to anti-human transferrin receptor and anti-mouse transferrin receptor antibodies by different methods (26, 30). We adopted a rapid thioether linkage technique to form strictly bispecific hybrids using Traut's reagent and the heterobifunctional SMBP reagent. One component was sparingly substituted with thiol groups (SH). These readily reacted to form a thioether linkage upon mixture with the maleimido-substituted (M) second component.

$$Ab_A\text{-}SH + Ab_B\text{-}M \rightarrow Ab_A\text{-}S\text{-}Ab_B$$

Gel filtration of the reaction mixture on an S-300 column yields the purified dimer which is 300kDa and has two sites for binding Aß plus two sites for attachment to transferrin receptors on brain capillary endothelial cells. $F(ab')_2$ fragments of the two different antibody types will be similarly thioether-linked to form Fc-devoid reagents that cannot bind complement which might otherwise cause neurotoxic effects.

Non-targeted control hybrids have been formed by linking a nonspecific MOPC antibody to the anti-Aß antibody. This hybrid antibody will bind Aß, but, being non-reactive with transferrin receptors, should not cross the blood-brain barrier.

Smaller bispecific hybrids (100kDa) have been formed by reducing the intrinsic disulfides which link the heavy chains of $F(ab')_2$ fragments (49). The thiols generated were stabilized and Ellman's reagent (E) was used to activate these groups on one of the components (50). Exclusively bispecific $F(ab')_2$ hybrids were formed upon mixing the reduced Fab' with an activated Fab' having the alternate specificity.

$$Fab'_A\text{-}SH + Fab'_B\text{-}SS\text{-}E \rightarrow Fab'_A\text{-}SS\text{-}Fab'_B + E\text{-}SH$$

Purification on an S-200 column provided hybrids with one site for binding Aß and one site for interaction with the target epitope on the brain capillary endothelial cells.

A similar approach can be used to make even smaller disulfide-linked single chain Fv heterobispecific dimers, $Fv_A\text{-}SS\text{-}Fv_B$ (50kDa), to cross the blood-brain barrier. Soluble Fvs have been constructed to possess a carboxyl-terminal cysteine to facilitate the disulfide exchange shown below and create 50kDa heterodimers exclusively.

$$Fv_A\text{-}SH + Fv_B\text{-}SS\text{-}E \rightarrow Fv_A\text{-}SS\text{-}Fv_B + E\text{-}SH$$

In side by side comparisons between whole antibody and either Fab' or Fv based bispecific reagents, the latter have proven to be moderately more effective on a molar basis for cell uptake via the transferrin receptor-mediated pathway. Since these smaller constructs are monovalent for the cell-surface epitope, those findings dispel the notion that cross-linking of two surface receptors is necessary for the cellular uptake of immunocomplexes.

c. Characterization of bispecific antibodies: After the synthesis, purification and size analysis of the anti-Aß/anti-transferrin receptor bispecific antibody was completed, a series of functional assays were performed. Its ability to attach to transferrin receptor bearing human cells was confirmed by cytofluorimetry using an anti-mouse IgG probe (Fig. 16). The capacity of the hybrid reagent to bind ^{125}I-Aß compared favorably with that of the parent anti-Aß antibody (Table III).

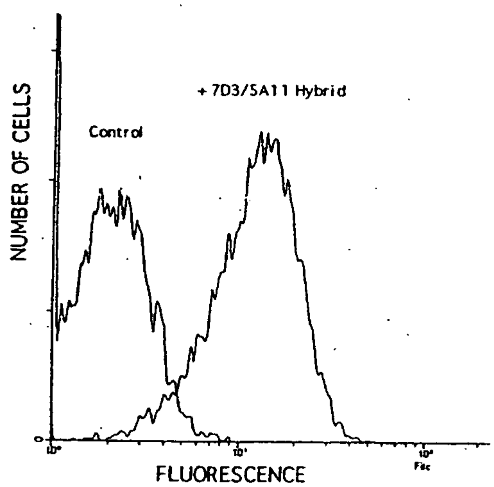


Table III **^{125}I-Aß Binding to Bispecific Antibody**

Addition	^{125}I-Aß$_{1-40}$ Bound (cpm)
Control	4,199
+ anti-Aß	23,301
+ anti-Aß/anti-receptor	22,850

Fig. 16 Attachment of Bispecific Antibody to Receptor-Positive Cells

To ensure that both of these binding activities resided on the bispecific antibody we treated transferrin receptor positive cells with the hybrid reagent, washed away unbound material and then exposed these cells to ^{125}I-Aß$_{1-40}$. The cells were washed and the amount of cell-bound radioactivity was compared to control cells which had been identically prepared except that pretreatment with bispecific antibody was omitted.

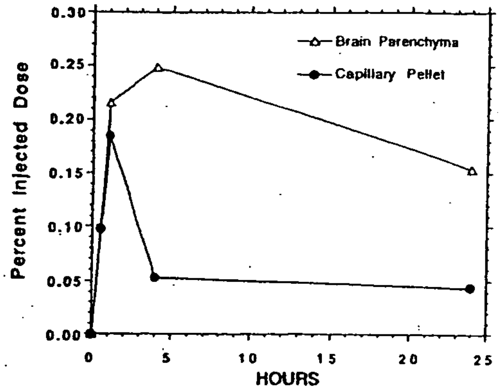
Table IV **Bispecific Antibody-Mediated Binding of ^{125}I-Aß to Receptor-Positive Cells**

Pretreatment of Cells	^{125}I-Aß$_{1-40}$ Bound (cpm)
None	2,367
+ anti-Aß/anti-transferrin receptor	11,476

The results (Table IV) verify the dual specificity of this bispecific antibody by clearly showing that it can simultaneously attach to the cell membrane and bind ^{125}I-Aß$_{1-40}$.

The vectorized bispecific antibody prepared for use in the transgenic mouse model of Alzheimer's disease is composed of a rat monoclonal antibody directed against the mouse transferrin receptor plus the 5A11 mouse anti-Aß monoclonal antibody. Both components were detected on the cell membrane by cytofluorimetry (Fig. 16) when this duplex was reacted with transferrin receptor positive mouse cells and probed using either a rat IgG-specific or mouse IgG-specific fluorescent secondary antibody reagent (data not shown).

d. Transcytosis of bispecific antibody into the brain: We coupled a rat monoclonal anti-mouse transferrin receptor antibody to a mouse monoclonal antibody so that the entry of this new vectorized bispecific construct into brain could be monitored. The bispecific antibody was labeled with ^{125}I and injected i.v. into normal mice. After different times the mice were sacrificed and the amount of ^{125}I-bispecific antibody which crossed the blood-brain barrier and entered the brain was determined by a mouse capillary depletion method (25, 51).



The amount of vectorized bispecific antibody found in the brain parenchyma or brain capillary fractions was measured following differential density centrifugation of the brain homogenate. These values were plotted as a function of time after i.v. injection (Fig. 17). The time-dependent redistribution of radiolabeled bispecific antibody from the capillaries and into the parenchyma is consistent with its passage across the cerebral endothelial blood-brain barrier (20). These preliminary experiments, which were performed in normal mice, will be repeated in plaque-bearing transgenic mice, when they reach 1-year of age. Greater accumulation in the parenchyma may result if the antibodies attach to Aß in the cerebral plaques.

Fig. 17 Transcytosis of the Vectorized Bispecific Antibody into Brain

e. Monitoring the brain distribution of bispecific antibody in live mice: An ability to follow the entry and accumulation of vectorized bispecific antibodies in the brain of live mice would assist in developing the intracerebral treatment of plaque-bearing mice. Time-course studies could be easily carried out and problems with inter-mouse variability would be greatly reduced. We therefore have begun preliminary studies with ^{125}I-labeled bispecific antibodies, to determine if immunoscintigraphy is feasible in this system. As a first step, we administered either the radiolabeled vectorized bispecific antibody (^{125}I-R17/5A11) or a non-vectorized control bispecific antibody to separate mice. Sequential brain images were accumulated at 1, 6, 24 and 48 hours following i.v. administration of the ^{125}I-labeled bispecific antibody probes. This technique suffers because it is difficult to determine how much of the signal is due to the levels of blood-borne radioactivity circulating through the brain. However distinctions were noted in the brain of mice treated with the mouse transferrin receptor reactive bispecific antibody versus those receiving the control bispecific antibody (Fig. 18, color images provided in the Appendix). When the vectorized agent was used, brain levels increased between 1 and 6 hrs and then declined to a much lower level at 24 and 48hrs. Mice treated with the control displayed no increase between 1 and 6 hrs. The reason for decreased brain levels at 24 hrs and beyond is not known but this might be due to dehalogenation of the bispecific antibody probes so that free ^{125}I is released and exits the brain. Alternative radioactive labels such as ^{111}In (52) or ^{99m}Tc (53) will be attached to the vectorized bispecific antibody if the use of iodine presents a technical problem.

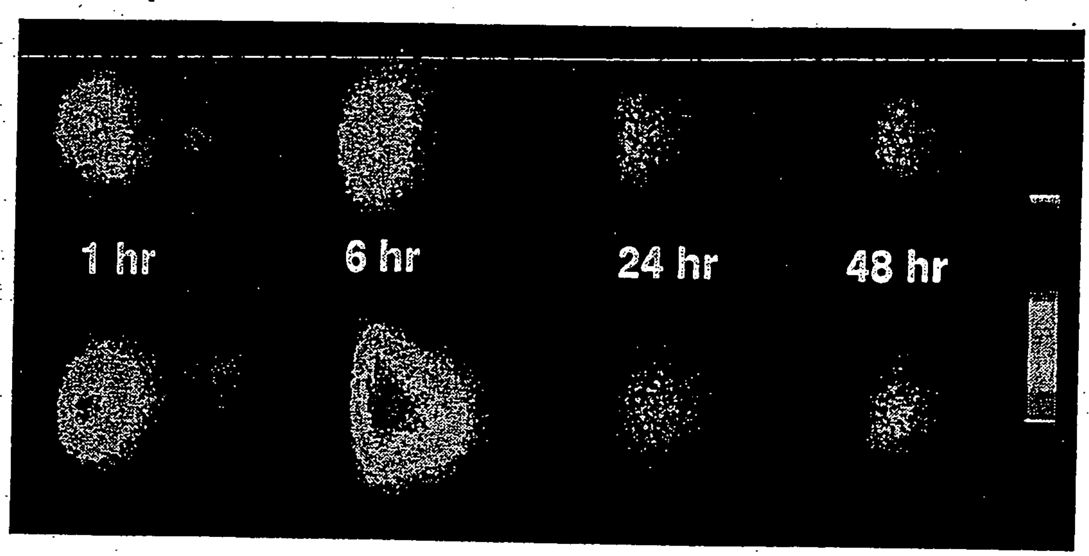


Fig. 18 Dorsal Aspect Brain Images of Mice (Nose to Right) Treated with Vectorized (bottom) or Control (top) Bispecific Antibody; Darker Central Region in the Image Denotes Greater CPM

Further studies and additional controls will be run to determine the significance of the apparent differential distribution of these ^{125}I-labeled probes. For example we will co-inject a large excess of unlabeled vector antibody (anti-mouse TfR) with the ^{125}I-labeled vectorized bispecific antibody in an attempt to block its receptor-mediated entry into the brain. This imaging technology will be useful for determining if smaller vectorized bispecific antibodies (eg. F(ab')$_2$) with different physical properties and an altered biodistribution will penetrate into the brain more effectively. Importantly, the digtital scintigraphy data shown above can be easily quantified using standards and the integration functions provided in the analysis software. For example, the central dark region in the brain image shown above corresponds to $\sim 1.5 \times 10^5$ cpm.

C.3 Disruption of Plaque Development in Transgenic Mice

a. The Tg2576 transgenic mouse colony: Transgene-positive Tg2576 mice for breeding were generously provided by Dr. Karen Hsiao (44). She provided us with the pertinent breeding information concerning these mice and has offered to answer questions if any problems arise. Our animal care technicians are well experienced in handling transgenic mice and have established a healthy colony.

Dr. Hsiao recommended that we perform genotyping on the Tg2576 mice both before and after each experiment. The 3'UT of the hamster cosmid PrP vector will be used as a hybridization probe (45) and we have received a sample of this region. Ear punch biopsy DNA has been prepared and PCR procedures have been carried out on every weaned mouse to identify mice bearing the transgene (45) (data not shown). We now routinely process and cut brain sections for immunocytochemical and thioflavin S detection of amyloid plaques in these mice (Fig. 19). Aβ in brain tissue will also be extracted and quantified by ELISA as previously described (45).

We currently have a large number of young transgene positive mice which are available for use to perform this ongoing research. Many one year-old experimental animals with preestablished amyloid plaques (Fig. 19) are also now ready for our proposed experimental studies on the cerebral delivery of anti-Aβ antibodies.

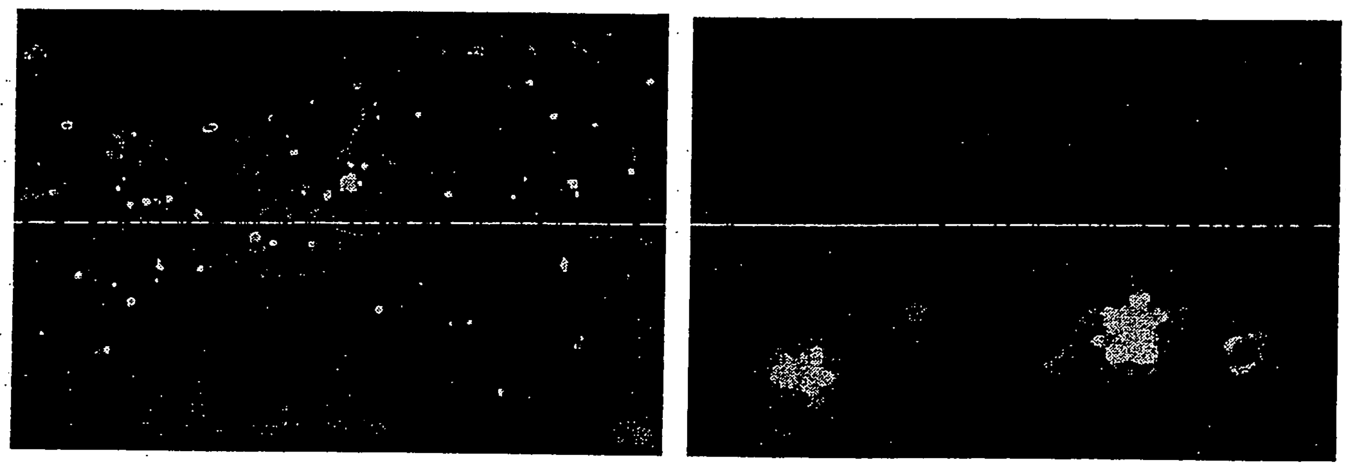


Fig. 19 *Low- and High-Power Micrograph of Thioflavin Stained Brain Plaques from Our Tg Positive Mice*

D. RESEARCH DESIGN and METHODS

D.1 Elicit Monoclonal Antibodies with Native Aβ and Transition State Aβ Antigens

a. Antigen synthesis, immunization and hybridoma fusion protocols: We have synthesized the Aβ 40mer, the three native Aβ peptides shown in Fig. 4 and the statine-based transition state Aβ peptides shown in Figs. 8 and 9 by standard automated Fmoc chemistry. A transition state phoshonamidate peptide (Figs. 5 and 7) was prepared by a combination of synthetic organic chemistry and automated Fmoc chemistry as outlined in Section C.1b. Synthesis of phosphonate transition state analogs will proceed along a similar line. Newly synthesized peptides will be purified by HPLC and their composition will be verified by mass spectral and amino acid analysis. The peptide synthesizer, mass spectrometer and amino acid analyzer are all located at our BBRI facility so that the synthesis of any additional Aβ peptides can be easily accomplished if and when they are needed.

The native and transition state Aβ peptides will be linked to antigenic carrier proteins like KLH or ovalbumin in order to elicit an immune response. A Cys residue will be strategically placed at the N or C-terminal end of the peptides to provide a suitable linkage group for coupling them via a thioether bond to maleimide activated carrier proteins. This stable linkage attaches the peptide in a defined orientation. Addition of ~20 peptides/KLH has been obtained based upon the transition state amino acid content as determined by amino acid analysis of the hydrolyzed conjugates.

The Aβ peptide antigens will be emulsified in complete Freunds adjuvant and injected i.p. into BALB/c mice. After ~1 month the animals will be given a boost i.p. using the antigen emulsified with incomplete Freunds adjuvant. Serum from these animals will be analyzed for anti-peptide antibodies by ELISA as shown in Preliminary Studies Section C.1g and C.1i. Animals showing abundant antibody production will be boosted by an i.v. injection with antigen and three days later they will be used to generate hybridoma clones that secrete monoclonal antibodies. Spleen cells from mice with the highest titre will be fused with mouse myeloma NS-1 cells to establish hybridomas according to standard procedures (54, 55). The production of monoclonal antibodies has been a mainstay of this laboratory for many years and fusions are routinely run on a bi-weekly basis. Preliminary Studies Section C.1 describes our progress toward obtaining anti-Aβ and anti-Aβ transition state analog monoclonal antibodies.

b. Screen and isolate select anti-Aβ antibodies: The initial screen for anti-Aβ and anti-transition state Aβ peptide monoclonal antibodies will be performed using ELISA (Preliminary Studies Section, C.1g and C.1i). Both the transition state peptide and the corresponding natural Aβ peptide will be adsorbed onto separate microtitre plates. The hybridoma supernatants will be screened using two assays so that the relative binding to both native and transition state Aβ peptides can be quantitated. Clones producing monoclonal antibodies that preferentially recognize the transition state or bind Aβ with high affinity will be selected for expansion and further study.

When searching for potential catalytic antibodies, the clones will be secondarily screened by ELISA to detect and discard antibodies with substantial affinity for the predicted cleavage products of the native Aβ peptide, eg. MVGG and VVIAT for the anti-phosphonate or anti-phosphoamidate antibodies; HQKLVF and FAEDVG for the anti-phenylalanine statine transition state antibodies; GGVV and IAT for the anti-statine antibodies. This is important since such catalytic antibodies would be subject to product inhibition and might therefore exhibit low turnover.

Our primary assay to screen hybridoma supernatants for proteolytic activity makes use of a solid phase ^{125}I-labeled Aβ peptide (Preliminary Studies Section, C.1h). The Sepharose-linked, ^{125}I-labeled Aβ substrate provides a very sensitive and convenient method for detecting catalytic cleavage by the progressive release of soluble ^{125}I-peptide. It is cleaved by several naturally occurring proteases but preliminary tests indicate that interference from high levels of background hydrolysis will not be a problem when assaying hybridoma supernatants of clones that do not produce catalytic antibodies

(Fig. 11). As a further precaution against exogenous proteases, all hybridoma cell fusions and cell culturing can be carried out in serum-free media.

General assays for detecting the proteolytic cleavage of any of the synthetic Aß peptides would include HPLC analysis or TLC followed by detection of the newly generated peptides using the highly sensitive fluorescamine reagent. High specific activity ^{125}I-Aß has been synthesized. Cleavage of ^{125}I-labeled Aß peptides will be discerned by autoradiography following separation by silica gel TLC using ethanol-10% conc. NH_4OH.

Several important variables will be examined for their impact on the catalytic activity of the anti-transition state antibodies, including pH, temperature, ionic strength and the presence of different metal ions. Thus for example, specific cleavage will be assayed at three pH values, pH 5, pH 7 and pH 9 either at 25^{o} or 37^{o} C. Zn^{++}, Cu^{++} and other metals that can facilitate peptide hydrolysis will be tested for effects on the antibody catalyzed reaction.

Selection of the highest affinity monoclonal anti-Aß antibodies will be accomplished by screening clones with the ^{125}I-Aß binding assay described in Preliminary Studies Section, C.1f. This assay is extremely sensitive and, by using serial dilution, will provide relative binding affinities for the different hybridoma supernatants or purified antibodies.

Identification of anti-Aß antibodies with the capacity to dissolve Aß aggregates will be performed using either a radioactive assay or a fluorescence-based microscopic assay. A description of those two procedures and examples of the data obtained are provided in Preliminary Studies Section, C.1j and Fig. 13 and Table II.

Judging by our preliminary criteria, we will determine which anti-Aß hybridoma clones to expand and use for ascites induction. Selected clones producing anti-Aß antibodies and clones producing anti-receptor antibodies will be injected into separate pristane-primed mice. Ascites will be collected and the specific monoclonal antibodies will be isolated. Purification can be accomplished using a Protein A column or antibodies can be isolated from ascites fluid by $(NH_4)_2SO_4$ precipitation and passage over an S-300 column to obtain the 150kDa immunoglobulin fraction. Monovalent Fab fragments will be prepared and isolated by established methods. Their purity will be evaluated by SDS-PAGE under reducing and non-reducing conditions.

Chronic treatment of transgenic Tg2576 mice biweekly with 1.5 mg of a vectorized bispecific antibody for 10 weeks will require 30 mg/mouse. We routinely obtain 50-100mg of purified monoclonal antibody from each ascites-bearing mouse. Thus an ample supply of these reagents will be available for use in our study of their effect on Aß depletion and behavioral deficits in Tg2576 mice.

With purified anti-Aß antibodies in hand, we will proceed to further characterize their binding specificity, the mechanism of antibody-dependent Aß cleavage as well as the resulting peptide cleavage products. Direct binding of ^{125}I-Aß peptides to the antibodies will be measured by equilibrium dialysis or by the polyethylene glycol precipitation method described in the Preliminary Studies Section. Competitive displacement assays will be used to measure the relative binding strength of different unlabeled Aß peptides. This assay will provide a quantitative measure of affinity that will augment the specificity data obtained by ELISA.

Many of the assays and conditions detailed in the Preliminary Studies Section for measuring catalytic activity on Aß substrates will be employed to fully define the hydrolytic properties of the isolated anti-transition state antibodies. As described, a comparison will be made with the uncatalyzed reaction run under identical conditions. At this stage, however, some very important controls will be run. First we will ensure that catalytic antibody activity is completely blocked by the appropriate transition state peptide. This non-cleavable "inhibitor" should bind much more tightly to the antibody combining sites and thereby prevent substrate binding or cleavage. Substrate specificity will be further established by showing no cleavage of a sham Aß peptides having a different amino acid sequence. The products of hydrolysis will be fully characterized by HPLC, amino acid and mass spectral analysis. Control antibodies that are not directed against the transition state Aß will be tested and are expected to produce no catalysis. Catalytic activity will be shown to reside in the purified Fab fragments of the anti-transition state antibody.

A kinetic analysis of the catalyzed reaction will be performed by measuring the initial rates of hydrolysis as a function of substrate concentration. Data will be analyzed on a Lineweaver-Burke plot so that it can be confirmed that the reaction follows classical Michaelis-Menten kinetics. A rate acceleration for hydrolysis will be calculated by the ratio of k_{cat}/k_{uncat}. The reaction will also be run for an extended length of time to demonstrate catalytic turnover.

In addition to demonstrating binding to and cleavage of soluble Aß peptides, we will also explore the effect of the purified anti-Aß antibodies on insoluble Aß precipitates (Preliminary Studies Section C.1j) (23, 24). A highly active catalytic antibody might destroy insoluble Aß plaques by hydrolytically cleaving the constituent aggregated peptides. Moreover, if insoluble peptide is in equilibrium with a low level of soluble Aß, then an anti-Aß binding antibody could upset this balance and gradually dissolve the precipitate. These interesting possibilities will be tested since Aß precipitates can be easily formed and measured *in vitro* (8, 9) (Fig. 17 and Table II).

c. Infuse anti-Aß antibodies directly into the CNS of mice: Anti-Aß antibodies which selectively cleave, or tightly bind the Aß peptide and/or dissolve its aggregates *in vitro* will be delivered into the brain of plaque-bearing Tg2576 transgenic mice (44) by direct intracerebral infusion. This technique will give these antibodies immediate access to Aß in the brain without having to cross the blood-brain barrier. Subsequent action of the antbodies on size and/or number of Aß brain plaques will then be monitored. Consequently, intracerebral infusion experiments will provide valuable preliminary data that would help us select anti-Aß antibodies which should produce the best vectorized bispecific reagents for affecting cerebral plaques.

A cannula must be stereotaxically placed in the skull of Tg2576 mice in order to facilitate direct parenchymal or intracerebroventricular infusion of our anti-Aß antibodies into the brain. This surgical procedure will initially be performed, for a modest fee, by surgical technicians at Taconic Technical Services. Briefly, the animal is anesthetized using sodium pentobarbital and placed in a stereotaxic frame. A midsaggital incision is made on the scalp to expose the skull, and the underlying fascia is scraped away. A hole is drilled to accept a sterilized length of stainless steel hypodermic tubing, which is stereotaxically advanced so that its tip is appropriately located in the brain. The guide cannula is then attached to the skull using a special glue and is sealed with a screw-on stylet.

Dr. M. J. Young routinely performs brain cannulation procedures here at our in-house BBRI animal facility. We have and will continue to consult with him regarding these infusion experiments. He has all of the required equipment for those studies and this gives us an good opportunity to learn the surgical techniques for cannula placement ourselves.

The cannula is expected to remain in place for a least two weeks so that we can administer multiple infusions of antibody into the brain of plaque-bearing Tg2576 mice. A 1 µl bolus of a sterile 50mg/ml solution (56) of either a monoclonal anti-Aß or irrelevant control antibody will be microinjected into immobilized mice via an injection cannula (infused over a 2-8 min period using a PE-10 fluid line connected to a 10 µl syringe driven by a syringe pump).

At the completion of an experiment the animal will be sacrificed and its brain will be fixed, frozen and sectioned for analysis. Alternate sections will be processed to reveal either the plaques (Fig. 18) or the distribution of infused antibody by immunohistochemical staining (56). We would expect to find fewer and/or smaller plaques in an area of the brain where the infused anti-Aß antibody spreads as opposed to regions which are devoid of anti-Aß (eg. the opposite hemisphere). Animals which receive an irrelevant control antibody should show no plaque reduction.

D.2 Produce Vectorized Anti-Aß/Anti-Receptor Bispecific Antibodies

a. Construct vectorized bispecific antibodies: The idea of producing hybrid antibodies with dual specificity was introduced in 1961 by Nisonoff and Rivers (57). They constructed reagents that simultaneously bound human and duck red blood cells to yield mixed hemagglutination. Many

years later my laboratory recognized that such reagents could serve as very useful biological tools. We showed that specially designed bispecific antibodies could be used to target drugs and toxins to the surface of selected cell types and then deliver these agents into the cytosol by receptor mediated endocytosis (29, 30, 58, 59). More recently we have developed bispecific antibodies which are triggered to release their payload into specific acidic subcellular compartments (26-28, 60). Importantly, one of the targets that we employed for those cellular studies is the same transferrin receptor that we plan to use in this proposed project for vectorized transcytosis across the blood-brain barrier. Thus, we have a long history involving the design, construction and use of bispecific antibodies for novel biological purposes.

Different schemes have been developed to unidirectionally couple whole antibodies or their antigen-binding fragments so that strictly heterobifunctional molecules are formed (see Preliminary Studies Section C.2b). Those coupling methods will be used to construct the different bispecific antibodies needed for this project. Typically an antibody with one specificity is chemically modified to render it reactive with sulfhydryl groups. Antibody with the alternate specificity is treated to either expose its intrinsic sulfhydryl groups or add extrinsic sulfhydryl groups. A cross-linking reaction occurs when these two components are mixed, resulting in the formation of either a disulfide or thioether bond. This chemistry is gentle and generally applicable to most antibodies but functional assays will be performed to ensure that the antibody binding activity is preserved (see the Research Design and Methods Section D.2c). Methods have been described for genetically engineering bispecific reagents or for producing them intracellularly by fusing the two different hybridoma clones. While such procedures may be advantageous at the latter stages of our project, they are not an expeditious way to produce the vectorized bispecific antibodies needed in the initial phase of this work.

b. Prepare $F(ab')_2$ heterodimers for vector-mediated transport into the brain: We will also produce smaller vectorized $F(ab')_2$ bispecific reagents to test in the transgenic mouse model for several important reasons. The introduction of whole antibodies into the brain might be detrimental if they were to fix complement and promote complement-mediated lysis of neuronal cells. It has been shown that aggregated Aß itself can fix complement in the absence of any antibody and that the resulting inflammation may contribute to the pathology of Alzheimer's disease (61-65). The possibility of intracerebal antibody having a similar effect would be greatly reduced by eliminating the Fc region of the antibody.

Faster or more efficient entry into the brain represents another potential advantage that smaller $F(ab')_2$ or Fv_2 reagents might provide for our intracerebral delivery strategy. We will therefore prepare those modified bispecific agents and compare them with full-sized hybrid antibodies for there relative effectiveness in reaching the brain, crossing the blood-brain barrier and affecting Aß plaque development (see the Research Design and Methods Section D.3 for details). It is important to note however that only minor differences were found when we compared the capacity of differently-sized anti-transferrin receptor bispecific reagents for delivering toxins into cells by receptor-mediated endocytosis (26). This observation might indicate that little variation will be seen for transcytosis across the brain capillary endothelial cells which form the blood-brain barrier. At the very least however we would expect the two types of vectorized molecules to have different biodistribution and plasma half-life characteristics (66).

c. Test the vectorized anti-Aß for dual specificity and entry into the brain: We will use several different functional assays to verify that both binding activities of our newly synthesized vectorized bispecific antibodies have remained intact. These include a cytofluorometic assay to monitor the attachment of the bispecific antibody to the surface of receptor-positive cells (see Preliminary Studies Section C.2c and Fig.15). In cases where the two halves of a hybrid molecule are derived from different species each component will be identified on the membrane using species-specific secondary fluoresent probes. The ability of the anti-Aß half to bind peptide will be measured using ^{125}I-Aß and a polyethylene glycol separation technique (see Preliminary Studies Section C.2c and Table III). To ensure bispecificity, each hybrid reagent will be tested for a capacity to mediate the attachment of ^{125}I-Aß to receptor-bearing cells (see Preliminary Studies Section C.2c and Table IV). This demonstrates unequivocally that both functional activities reside on the same duplex molecule.

The capacity of each vectorized bispecific antibody preparation for transcytosis across the blood-brain barrier and entry into the brain will be evaluated by the capillary depletion method described in the Preliminary Studies Section C.2d and Fig. 16. We will utilize the radiolabeled bispecific antibody injected i.v. into mice and measure its distribution into the parenchymal and capillary fractions of the brain homogenate as function of time. Progressive passage from the capillaries and into the parenchyma will be indicative of active transcytosis across the blood-brain barrier.

A supplementary, non-invasive method for monitoring this intracerebral delivery process is currently being explored and shows promise for future studies. This involves visualizing the entry of a radiolabeled bispecific antibody into the brain of live mice using immunoscintigraphy (see Preliminary Studies Section C.2e and Fig. 17).

D.3 Test Vectorized Bispecific Antibodies for Disrupting Plaque Development in Mice

a. Incoporation of ^{125}I-Aß into amyloid plaques: It has been shown that i.v. injected ^{125}I-Aß enters the brain (67). Moreover, *in vitro*, ^{125}I-Aß binds specifically, reversibly and with high affinity to preformed amyloid plaques in unfixed brain sections and homogenates obtained from postmortem Alzheimer's disease patients (13). Taken together, these two observations suggest that i.v. injected ^{125}I-Aß might serve as a good, quantitative probe for following amyloid plaque development *in vivo* using a transgenic mouse model of Alzheimer's disease (44).

To test whether the proposed *in vivo* assay does work, brain tissue from ~1 year old transgenic mice with plaques will be compared to brain tissue from 2-8 month old mice without plaques (44). As a first step, we will measure *in vitro* binding of ^{125}I-Aß directly to plaques in brain homogenates as previously described (13). This procedure uses a glass fiber filter to trap the plaques and any radioactive Aß that has associated with them. It is expected that homogenates from older mice that have plaques will bind significantly more ^{125}I-Aß than those from young transgenic mice that are plaque-free.

For the *in vivo* assay, the two groups of mice (i.e. young and old transgenic mice) would receive ^{125}I-Aß intravenously. After a set interval to allow the ^{125}I-Aß to reach and bind to plaques (eg. 5h or 24h) the animals will be scarificed. Brain tissue will be removed, homogenized and the amount of ^{125}I-Aß bound to plaques measured by the glass fiber filter assay (13). Homogenates from the older mice should bind significantly more of the i.v. administered ^{125}I-Aß than those from the young transgenic mice which have not developed plaques. As a specificity control, certain plaque-bearing mice will receive an excess of unlabeled Aß co-injected with the ^{125}I-Aß. This should effectively block the specific binding of ^{125}I-Aß to plaques (13). The specificity of the interaction will be further verified by using autoradiography of brain sections to localize the ^{125}I-Aß to plaques in the cortex (13).

b. Incoporation of ^{125}I-labeled bispecific antibodies into amyloid plaques: Many anti-Aß monoclonal antibodies have been shown to bind to Aß plaques in tissue sections, making it reasonable to expect that a similar reaction could occur when vectorized anti-Aß antibodies are administered *in vivo* and enter the brain. We will routinely test our antibodies for reaction with insoluble Aß aggregates produced *in vitro*. Some antibodies might react exclusively with soluble Aß but a few should also bind to these artificial plaques. One of those anti-Aß antibodies will be radiolabeled with ^{125}I and then coupled to an unlabeled anti-transferrin receptor antibody. This ^{125}I -labeled vectorized bispecific antibody probe will be administered i.v. to 1-year old, plaque-bearing transgenic mice so that the specific localization of ^{125}I-anti-Aß to cerebral plaques can be visualized using autoradiography of brain sections. Accumulation of these probes in the brain of live plaque-bearing mice will also be evaluated by immunoscintography (see Preliminary Studies Section C.2e and Fig. 17).

To test for the proposed *in vivo* localization, brain tissue from ~1 year old transgenic mice with plaques will be compared to brain tissue from 2-8 month old mice without plaques (44). First we will

measure *in vitro* binding of ^{125}I-anti-Aß directly to plaques in brain homogenates as previously described (13). This procedure uses a glass fiber filter to trap plaques and any radioactive antibody that has associated with them. It is expected that homogenates from older mice that have plaques will bind significantly more ^{125}I-anti-Aß than those from young transgenic mice that are plaque-free.

For the *in vivo* assay the two groups of mice (i.e. young and old transgenic mice) would receive ^{125}I-labeled vectorized bispecific antibody intravenously. After a set interval to allow the ^{125}I-probe to reach and bind to plaques (eg. 5h or 24h), the animals will be scarificed. Brain tissue will be removed, so that half can be used for autoradiography and the other half homogenized to measure the amount of ^{125}I-bispecific anti-Aß bound to plaques using the glass fiber filter assay (13). Homogenates from the older mice should bind significantly more of the i.v. administered ^{125}I-bispecfic anti-Aß than those from the young transgenic mice which have not developed plaques. The specificity of the interaction will be further verified by using autoradiography of brain sections to localize the ^{125}I-bispecific anti-Aß to plaques in the cortex (13). Gross accumulation of this probe in the brain of live transgenic mice will also be visualized by immunoscintography (Fig. 17). As a delivery control, certain plaque-bearing mice will receive an excess of unlabeled anti-transferrin receptor antibody co-injected with the ^{125}I-bispecific anti-Aß. This should effectively block the transport of the ^{125}I-bispecific anti-Aß into the brain and prevent the labeling of plaques. Further negative controls will use ^{125}I-bispecific antibodies in which either the anti-Aß or anti-transferrin half is replaced by an irrelevant antibody.

c. Bispecific antibody inhibition of ^{125}I-Aß incorporation into amyloid plaques: The ^{125}I-Aß *in vivo* probe will allow us to address two pivotal questions. Can vectorized bispecific anti-Aß antibodies prevent the deposition of ^{125}I-Aß into brain plaques; and, more importantly, will they facilitate the removal of ^{125}I-Aß that has already been deposited into the plaques?

To answer the first question, a vectorized bispecific anti-Aß antibody or a non-specific control bispecific antibody (1-5mg/300µl) will injected intravenously into separate groups of older plaque-bearing transgenic mice. An appropriate interval will be allowed so that the level of bispecific antibody will rise in the brain and will be depleted in the blood. Then ^{125}I-Aß will be administered i.v. to these animals so that, they can be sacrificed and tested for ^{125}I-Aß incorporation into brain plaques as described above in Section D.3a. We would predict that the presence anti-Aß antibodies in the brain would prevent ^{125}I-Aß from labeling brain plaques by virtue of their ability to cleave or sequester this ^{125}I-peptide. Plaques from mice treated with the non-vectorized control antibody, in contrast, should show substantial *de novo* incorporation of ^{125}I-Aß.

The second part of this study will be carried out by pre-injecting ^{125}I-Aß into plaque-bearing transgenic mice so that the radiolabeled peptide can incorporate normally into the amyloid plaques. Preferably, a period of time will be allowed so that the level of ^{125}I-Aß in the blood of these mice is lowered. Animals with these pre-labeled plaques will then be given an i.v. injection containing 1-5mg/300µl of the vectorized bispecific anti-Aß antibody or a non-vectorized control bispecific antibody. Mice from these two treatment groups will be sacrificed after different time intervals and tested for the amount of ^{125}I-Aß that remains incorporated within brain plaques as described above. Very slow dissociation of plaque-bound ^{125}I-Aß is expected for the mice treated with non-specific control antibodies. The vectorized catalytic anti-Aß antibodies and vectorized anti-Aß binding antibodies however might greatly accelerate removal of the ^{125}I-Aß from plaques by acting as a sink to displace the Aß equilibrium away from the plaque. As a further control, some of the animals with pre-labeled ^{125}I-Aß-plaques will be injected i.v. with an excess of unlabeled Aß. This "blocking" control should prevent the re-association of ^{125}I-Aß that has already dissociated from the plaques.

d. Effect of bispecific anti-Aß on amyloid plaques and memory impairment: The age-dependent appearance of neurological symptoms in the Tg2576 transgenic mouse model offers some important opportunities for experimental intervention. Mice less then 9-months old had low levels of $Aß_{1-40}$ and $Aß_{1-43}$ in the brain, 48pmol/gm and 13pmol/gm, respectively, no amyloid plaques and good performance in the learning and memory tests (44). In contrast, mice over 9-months old had high brain levels of $Aß_{1-40}$ and $Aß_{1-43}$ (264pmol/gm and 175pmol/gm, repectively), many Aß plaques, and poor performance in learning and memory tests (56). We will administer

vectorized anti-Aß bispecific antibodies to young mice which show no abnormalities in order to prevent the changes which would inevitably occur at a latter age if they remained untreated.

Different groups of transgenic mice will be injected i.p. with 1mg of either vectorized bispecific anti-Aß antibodies or non-specific control bispecific antibodies, biweekly during months 7 through 10. Since the half-life of an injected homologous immunoglobulin is ~40h (30, 66) this protocol should maintain adequate anti-Aß antibody levels over that period of time. Little or no immune reaction would be expected since soluble immunoglobulins from the same species are poor antigens.

At the begining of month 11 and on consecutive months thereafter, some of the mice from each group will be tested for spatial reference learning and memory in the Morris water maze (44) and then they will be sacrificed to measure $Aß_{1-40}$ and $Aß_{1-43}$ concentrations and the number of amyloid plaques in the brain (44). My laboratory is well trained in the required immunocytochemical and ELISA techniques. The characteristics for each of the experimental procedures are provided in great detail by Hsiao et al (44) and, for the sake of consistency, will be followed as closely as possible. The maze will be constructed according to specifications (44) in the shop at BBRI and animal trials will be recorded on videotape for subsequent analysis. We will use normal mice to gain experience in this new area of behavioral research prior to performing experiments with Tg2576 mice. The escape latency data will be examined with a multifactor analysis of variance including genotype, age, and training day to reveal significant main effects. $Aß_{1-40}$ and $Aß_{1-43}$ concentrations will be measured by ELISA. Mouse brain sections for immunocytochemical and thioflavin S detection of amyloid plaques (Fig. 18) will be processed and evaluated, with our supervision, in a double-blind manner by the highly qualified, in-house Morphology Unit. The histology technician who will prepare specimens is HTL ASCP registered and has 7-years experience working in a neuropathology lab.

If the vectorized catalytic or binding anti-Aß antibodies displace Aß equilibria away from brain plaques, then transgenic mice from this group might show a delay or abrogation of symptoms. Compared to the non-specific control bispecific antibody treated mice, these animals could have reduced concentrations of $Aß_{1-40}$ and $Aß_{1-43}$ in the brain and fewer or smaller amyloid plaques. If these changes in Aß do occur and the mice also show improved memory and learning compared to controls, then the results would strengthen the correlation between soluble Aß, the plaques it forms and neurologic impairment.

In addition to testing the vectorized anti-Aß antibodies for preventing the formation of amyloid plaques and the development of memory deficits in young mice, we will examine the effects of these agents on older transgenic mice which are already affected. Different groups of transgenic mice will be injected i.p. with 1mg of either vectorized anti-Aß antibodies or non-vectorized control bispecific antibodies, biweekly during months 11-12.

At the begining of month 13 the mice from each group will be tested for spatial reference learning and memory in the Morris water maze (44) and then they will be sacrificed to measure $Aß_{1-40}$ and $Aß_{1-43}$ concentrations and the number of amyloid plaques as outlined above (44).

These experiments with older mice are designed to test the possibility of reversing the symptoms in animals which already have elevated $Aß_{1-40}$ and $Aß_{1-43}$ and amyloid plaques in the brain as well as neurologic deficits. If the vectorized anti-Aß bispecific antibodies displace Aß equilibria away from plaques in the brain then transgenic mice from that group might show a reduction or abrogation of symptoms. Compared to the non-specific control bispecific antibody treated mice, the concentrations of $Aß_{1-40}$ and $Aß_{1-43}$ in the brain of these mice might be reduced and they may have fewer or smaller amyloid plaques. If these changes in Aß do occur and the animals also show improved memory and learning compared to controls, then the results would strengthen the correlation between soluble Aß, the plaques it forms and neurologic impairment. If there is a significant reduction in amyloid plaques but no memory improvements, then this would indicate either no causal relationship between plaques and neurologic deficits or simply that some secondary damage which had already done to the brain is irreversible.

e. Monitoring animals for adverse effects due to treatment: Throughout the course of these experiments mice will be observed for any complications due to treatment with the vectorized bispecific antibodies. We will look for overt signs of immune complex disease, anaphylaxis or

neurological episodes. Manifestations in the mouse would be respiratory distress, emphysema, asphyxia and/or heart or kidney failure. Signs of neurological involvement may include seizures, paralysis, erratic behavior or wasting. It should be noted however that these possibilities are extremely remote since we will be using homologous mouse antibodies in an experimental mouse model. Furthermore, the use of single epitope monoclonal antibodies, the small size of the Aß peptide and the large excess of antibody over Aß in the circulation should preclude the formation of precipitating immune complexes. Importantly, vector antibodies alone appear safe since animals dosed daily for two weeks with an anti-transferrin receptor antibody showed no loss of integrity of the blood-brain barrier using a radioactive sucrose probe (31). Lastly, none of the "hybridoma" mice that we immunized with Aß antigens or the anti-Aß ascites-producing mice displayed ill effects even though some of those induced antibodies cross-react with mouse Aß and mouse amyloid precursor protein.

There is a possibility that an immune response will develop against the covalent cross-linking substitutions used to create the bispecific antibodies. Therefore, the mice will be monitored for any sign of these potential complications. In chronically treated mice, this event might produce adverse immune complex affects and/or inactivation of the bispecific reagents by antibody blocking. We have tried to minimize these problems by sparingly substituting the whole antibodies with SPDP, which yields an innocuous, 4 atom aliphatic linker between the two antibodies. In the case of bispecific $F(ab')_2$ molecules the disulfide linkage is between intrinstic cysteine residues and therefore no extrinsic chemical modifications should remain.

Time table for "CEREBRAL DELIVERY OF VECTORIZED ANTI-ß-AMYLOID ANTIBODY"

Specific Aim A1 (Elicit Monoclonal Antibodies with Native Aß and Transition State Aß Antigens) is well underway. The synthesis and characterization of both native Aß and the new transition state analog Aß antigens is essentially complete. Immunization with those antigens has already produced several very interesting monoclonal anti-Aß antibodies and this has allowed us to proceed with Specific Aims A2 and A3. We now also have an opportunity to test the effects of these antibodies on Aß plaques *in vivo* following direct intracerebral infusion into transgenic mice. Additional new anti-Aß antibodies will be elicited and examined for novel features such as catalyic cleavage of the Aß peptide during Years 1-2.

Specific Aim A2 (Produce Vectorized Anti-Aß/Anti-Receptor Bispecific Antibodies) is proceeding and two vectorized monoclonal bispecific antibodies have already been constructed for potential use in humans and for the transgenic mouse model. However, additional hybridoma fusions may be needed to obtain the very best catalytic and/or high-affinity anti-Aß antibodies for altering Aß levels in the brain. Likewise, alternative vector antibodies (eg. anti-insulin receptor) will be used to construct supplementary bispecific reagents. The majority of this work on new vectorized anti-Aß antibodies will be carried out during Years 1-2.

Specific Aim A3 (Test Anti-Aß Bispecific Antibodies for Disrupting Plaque Development in Mice) gets to the heart of the matter and therefore every effort will be made to move these experiments forward as quickly as possible. We have the appropriate vectorized bispecific antibodies to begin experiments in transgenic mice. Our transgenic mouse colony is rapidly expanding so that we will soon be able perform these trials in a decisive manner. A training period has been allotted early in the project to allow us to become proficient at testing animals for spatial reference learning and memory in the Morris water maze. The maze will be constructed according to specifications in the shop at BBRI so that we can gain ample experience. Our plans to use the in-house Morphology Unit for histology services should significantly expedite tissue preparation and the analysis of Aß plaques in mouse brain sections. The studies outlined in this specific aim are expected to encompass Years 1-4. Year 4 is also requested to allow us to recognize and correct any unforeseen problems that might arrise. During Year 4 we will modify our best vectorized anti-Aß reagents for future use in non-human primate trials.

E. HUMAN SUBJECTS None

F. VERTEBRATE ANIMALS

The BBRI-ERI Joint Animal Facilities conform to Federal Guidelines (Guide for the Care and Use of Laboratory Animals) and has NIH approval (Sept. 1991-Jan.1996). The Animal Care and Use Committee reviews all protocols annually.

The animals used for this project include Balb/c mice, 6-10 weeks old mice and transgenic Tg2576 mice, 2-13 months old. The approximate number of animals to be used in the first 12-month period is as follows: 100 Balb/c mice and 100 Tg2576 mice. The Balb/c mice are required for the generation of anti-Aß monoclonal antibodies, which is an ongoing need for this research. The transgenic Tg2576 mice are needed to perform the radioactive Aß and vectorized bispecific antibody localization studies and to test the effect of passively administered vectorized bispecific anti-Aß antibodies on plaque formation and on the development of memory deficits. The restraining cages used during injections are comfortable for the animals and not overly restrictive.

Inhalation of carbon dioxide is the method of euthanasia which will be used. This method is consistent with recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

Veterinary and diagnostic services are provided by the Tufts University School of Veterinary Medicine in Jamaica Plain, Massachusetts through contract with the Angell Memorial Hospital, Robert Hopkins, DVM.

The principal investigator, laboratory personnel, and animal technicians involved in the proposed research have had instruction or have demonstrated their competence in the care, use and handling of laboratory animals. We give assurance of humane practice in animal maintenance and experimentation; and we subscribe to the concept of using every acceptable method in the performance of this project to minimize the use of animals and to prevent animal distress.

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65. Webster, S., B. Bradt, J. Rogers, and N. Cooper. 1997. Aggregation state-dependent activation of the classical complement pathway by the amyloid beta peptide. *J Neurochem* 69, no. 1:388-98.

66. Spiegelberg, H.L., and W.O. Weigle. 1965. The catabolism of homologous and heterologous 7S globin fragments. *J. Exp. Med.* 121:323.

67. Maness, L.M., W.A. Banks, M.B. Podlisny, D.J. Selkoe, and A.J. Kastin. 1994. Passage of human amyloid β-protein 1-40 across the murine blood-brain barrier. *Life Sciences* 55:1643-1650.

H. CONSULTANTS None

I. CONTRACTUAL ARRANGEMENTS None

CHECKLIST

TYPE OF APPLICATION *(Check all that apply.)*

__ NEW application. *(This application is being submitted to the PHS for the first time.)*

X REVISION of application number: 1 R01 AG16300-01
(This application replaces a prior unfunded version of a new, competing continuation, or supplemental application.)

__ COMPETING CONTINUATION of grant number: ______
(This application is to extend a funded grant beyond its current project period.)

INVENTIONS AND PATENTS *(Competing continuation appl.only)*
__ No
__ Yes. If "Yes," __ Previously reported
__ Not previously reported

__ SUPPLEMENT to grant number: ______
(This application is for additional funds to supplement a currently funded grant.)

__ CHANGE of principal investigator/program director.
Name of former principal investigator/program director: ______

__ FOREIGN application or significant foreign component.

1. ASSURANCES/CERTIFICATIONS

The following assurances/certifications are made and verified by the signature of the Official Signing for Applicant Organization on the Face Page of the application. Descriptions of individual assurances/certifications begin on page 27 of Section III. If unable to certify compliance where applicable, provide an explanation and place it after this page.

•Human Subjects; •Vertebrate Animals; •Debarment and Suspension; •Drug-Free Workplace *(applicable to new [Type 1] or revised [Type 1] applications only)*; •Lobbying; •Delinquent Federal Debt; •Research Misconduct; •Civil Rights (Form HHS 441 or HHS 690); •Handicapped Individuals (Form HHS 641 or HHS 690); •Sex Discrimination (Form HHS 639-A or HHS 690); •Age Discrimination (Form HHS 680 or HHS 690); •Financial Conflict of Interest.

2. PROGRAM INCOME *(See instructions, page 20.)*
All applications must indicate whether program income is anticipated during the period(s) for which grant support is requested. If program income is anticipated, use the format below to reflect the amount and source(s).

Budget Period	Anticipated Amount	Source(s)

3. INDIRECT COSTS

Indicate the applicant organization's most recent indirect cost rate established with the appropriate DHHS Regional Office, or, in the case of forprofit organizations, the rate established with the appropriate PHS Agency Cost Advisory Office. If the applicant organization is in the process of initially developing or renegotiating a rate, or has established a rate with another Federal agency, it should, immediately upon notification that an award will be made, develop a tentative indirect cost rate proposal. This is to be based on its most recently completed fiscal year in accordance with the principles set forth in the pertinent *DHHS Guide for Establishing Indirect Cost Rates*, and submitted to the appropriate DHHS Regional Office or PHS Agency Cost Advisory Office. Indirect costs will *not* be paid on foreign grants, construction grants, grants to Federal organizations, grants to individuals, and conference grants. Follow any additional instructions provided for Research Career Awards, Institutional National Research Service Awards, and specialized grant applications.

[X] DHHS Agreement dated: January 27, 1998 [ ] No Indirect Costs Requested

[ ] DHHS Agreement being negotiated with ______ Regional Office

[ ] No DHHS Agreement, but rates established with ______ Date ______

CALCULATION* *(The entire grant application, including the Checklist, will be reproduced and provided to peer reviewers as confidential information. Supplying the following information on indirect costs is optional for forprofit organizations.)*

a. Initial budget period: Amount of base $ 163,108 x Rate applied 92 % = Indirect Costs (1) $ 150,059

b. Entire proposed project period: Amount of base $ 692,632 x Rate applied 92 % = Indirect Costs (2) $ 637,221

(1) Add to total direct costs from form page 4 and enter new total on Face Page, Item 7b.
(2) Add to total direct costs from form page 5 and enter new total on Face Page, Item 8b.

*Check appropriate box(es):

[X] Salary and wages base [ ] Modified total direct cost base [ ] Other base (Explain)

[ ] Off-site, other special rate, or more than one rate involved *(Explain)*

Explanation *(Attach separate sheet, if necessary.)*:

4. SMOKE-FREE WORKPLACE

Does your organization currently provide a smoke-free workplace and/or promote the nonuse of tobacco products or have plans to do so?

[X] Yes [ ] No *(The response to this question has no impact on the review or funding of this application.)*

AA

Form Approved Through 9/30/97
OMB No. 0925-0001

Department of Health and Human Services
Public Health Service

Grant Application

Follow instructions carefully.
Do not exceed character length restrictions indicated on sample.

LEAVE BLANK-FOR PHS USE ONLY.

Type	Activity	Number
Review Group	Formerly	
Council/Board (Month, Year)		Date Received

1. TITLE OF PROJECT (*Do not exceed 56 characters, including spaces and punctuation.*)
NOVEL TRANSITION STATE PEPTIDE ANALOG ANTIGENS

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT [X] NO [ ] YES (*If "Yes," state number and title*)
Number: Title:

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR

3a. NAME (*Last, first, middle*): RASO, VICTOR A.
3b. DEGREE(S): PH.D.
3c. SOCIAL SECURITY NO.:
3d. POSITION TITLE: SENIOR SCIENTIST
3e. MAILING ADDRESS (*Street, city, state, zip code*):
BOSTON BIOMEDICAL RESEARCH INST.
20 STANIFORD STREET
BOSTON, MA 02114-2500
E-MAIL ADDRESS: RASO@BBRI.ORG
3f. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT:
3g. MAJOR SUBDIVISION:
3h. TELEPHONE AND FAX (*Area code, number and extension*)
TEL: (617) 912-0316
FAX: (617) 912-0306

4. HUMAN SUBJECTS [X] No [ ] Yes
4a. If "Yes," Exemption no. or IRB approval date [ ] Full IRB or [ ] Expedited Review
4b. Assurance of compliance no.

5. VERTEBRATE ANIMALS [ ] No [X] Yes
5a. If "Yes," IACUC approval date: 02/02/96
5b. Animal welfare assurance no.: A3177-01

6. DATES OF PROPOSED PERIOD OF SUPPORT (month, day, year-MM/DD/YY)		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)
12/01/99	11/30/04	175,428	302,251	928,650	1,615,564

9. APPLICANT ORGANIZATION
Name
Address
BOSTON BIOMEDICAL RESEARCH INST.
20 STANIFORD STREET
BOSTON, MA 02114-2500

10. TYPE OF ORGANIZATION
Public: → [ ] Federal [ ] State [ ] Local
Private: → [X] Private Nonprofit
Forprofit: → [ ] General [ ] Small Business

11. ORGANIZATIONAL COMPONENT CODE: 60

12. ENTITY IDENTIFICATION NUMBER: 1042451939A1
Congressional District: 9

13. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
Name: THOMAS J. MCQUAID
Title: ASSISTANT DIRECTOR
Address: BOSTON BIOMEDICAL RESEARCH INST.
20 STANIFORD STREET
BOSTON, MA 02114-2500
Telephone: (617) 912-0301
FAX: (617) 912-0335
E-Mail Address: MCQUAID@BBRI.ORG

14. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name: KATHLEEN G. MORGAN, PH.D
Title: DIRECTOR
Address: BOSTON BIOMEDICAL RESEARCH INST.
20 STANIFORD STREET
BOSTON, MA 02114-2500
Phone: (617) 912-0330
FAX: (617) 227-6053
E-Mail Address: MORGAN@BBRI.ORG

15. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PI/PD NAMED IN 3a. (*In ink. "Per" signature not acceptable.*)
Victor Raso
DATE: 1-27-99

16. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 14. (*In ink. "Per" signature not acceptable.*)
KG Morgan
DATE: 1-28-99

DESCRIPTION. State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This description is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. **DO NOT EXCEED THE SPACE PROVIDED.**

Therapies based upon catalytic antibodies hold great potential for the treatment of human afflictions like Alzheimer's disease, AIDS or drug addiction. This stems from the fact that catalytic antibodies constitute exquisitely specific reagents which can permanently alter or inactivate their targets. Moreover, since they exhibit turnover, each catalytic antibody will continuously destroy many target molecules rather than just binding stoichiometrically. Most catalytic antibodies are induced by immunizing with a transition state analog antigen. However, conventional transition state strategies have not yielded catalytic antibodies that will readily hydrolyze the peptide bond.

From a therapeutic standpoint, catalytic antibodies which can specifically cleave polypeptides would constitute a very important class of clinical reagents. They could irreversibly inactivate viral envelope proteins, bacterial toxins, enzymes or disease causing polypeptides within the body. Therefore, this proposed research tests the hypothesis that efficient proteolytic antibodies can be elicited by using elongated transition state peptide analogs having novel statine, reduced peptide bond or phosphonate moieties inserted into their amino acid sequence. The availability of transition state antigens which reliably elicit catalytic antibodies could establish a new paradigm in therapeutic vaccine design.

To this end, transition state peptides that closely resemble beta-amyloid or gp41 epitopes have been synthesized for potential use in combating Alzheimer's disease and AIDS, respectively. These surrogate peptides are designed to induce highly specific antibody combining sites that will stabilize the energetically unfavorable, rate-limiting, tetrahedral intermediate for peptide hydrolysis. The haptenic peptides were linked to carrier proteins, used to immunize mice and hybridomas were established. Binding assays have allowed selection of monoclonal antibodies that preferably recognize transition state peptides versus the native peptide or its predicted cleavage products. Several assays have been constructed to screen hybridoma supernatants for specific proteolytic activity. Select antibodies will be purified and characterized with regard to their catalytic activity. The transition state antigens produced may serve as a model for developing new clinical vaccines that establish a resistance to, or provide a treatment for disease by inducing catalytic antibodies rather than conventional neutralizing antibodies.

PERFORMANCE SITE(S) *(organization, city, state)*

BOSTON BIOMEDICAL RESEARCH INST
20 STANIFORD STREET
BOSTON, MA 02114

KEY PERSONNEL. See instructions on Page 11. *Use continuation pages as needed* to provide the required information in the format shown below.

Name	Organization	Role on Project
Victor Raso	Boston Biomedical Research Institute	Principal Investigator
Christine Kearney	Boston Biomedical Research Institute	Research Technician

Type the name of the principal investigator/program director at the top of each printed page and each continuation page. (For type specifications, see instructions on page 6.)

RESEARCH GRANT

TABLE OF CONTENTS

*Type density and type size of the entire application must conform to limits provided in instructions on page 6.

Appendix *(Five collated sets. No page numbering necessary for Appendix)* [X] Check if Appendix is Included

Number of publications and manuscripts accepted or submitted for publication *(not to exceed 10)* 5

Other items *(list)*:

Figure 5
Figure 10
Figure 11
Figure 12
Figure 14
Figure 17

Number pages consecutively at the bottom throughout the application. Do not use suffixes such as 3a. 3b.

Principal Investigator/Program Director *(Last, first, middle)*: Raso, Victor A.

DETAILED BUDGET FOR INITIAL BUDGET PERIOD DIRECT COSTS ONLY

FROM 12/1/99 THROUGH 11/30/04

PERSONNEL *(Applicant organization only)* NAME	ROLE ON PROJECT	TYPE APPT. (months)	% EFFORT ON PROJ.	INST. BASE SALARY	DOLLAR AMOUNT REQUESTED *(omit cents)* SALARY REQUESTED	FRINGE BENEFITS	TOTALS
Victor A. Raso	Principal Investigator	12.0	30	$109,153	$32,746	$9,496	$42,242
Katherine Sheldon	Res Assoc	12.0	100	$42,950	$42,950	$12,456	$55,406
Christine Kearney	Res Tech	12.0	100	$27,515	$27,515	$7,979	$35,494
Angela J. DiPerri	Admin. Assist.	12.0	10	$36,500	$3,650	$1,059	$4,709
SUBTOTALS					$106,861	$30,990	$137,851

Category	Items	Total
CONSULTANT COSTS		
EQUIPMENT *(Itemize)*	Liquid Nitrogen Tank $3,338; DEAE memsep column $1,500; ProA memsep column $575; 12-channel pipetter $650	$6,063
SUPPLIES *(Itemize bv*	Tissue culture sup. (media,sera,etc.) $7,500; Glassware & Disposables $1,500; Consumable Lab Supplies $2,000; Radiochemicals $1,500; Immunochemicals $2,000; Chromatography matls. $2,500; Chemistry sup./reagents $2,500; 100 mice @ 7.14 $714	$20,214
TRAVEL	Conference	$1,800
PATIENT CARE COSTS	INPATIENT	
	OUTPATIENT	
ALTERATIONS AND RENOVATIONS *(Itemize by category)*		
OTHER EXPENSES *(Itemize by category)*	Publ. Costs $1,000; Animal Housing $8,500	$9,500
SUBTOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD		$ 175,428
CONSORTIUM/CONTRACTUAL COSTS	DIRECT COSTS	
	INDIRECT COSTS	
TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD *(Item 7a, Face Page)*	→	$ 175,428

BUDGET FOR ENTIRE PROPOSED PERIOD OF SUPPORT
DIRECT COSTS ONLY

BUDGET CATEGORY TOTALS	INITIAL BUDGET PERIOD (from Form Page 4)	ADDITIONAL YEARS OF SUPPORT REQUESTED 2nd	3rd	4th	5th
PERSONNEL: *Salary and fringe benefits Applicant organization only*	$137,851	$143,365	$149,100	$155,064	$161,266
CONSULTANT COSTS					
EQUIPMENT	$6,063	$1,500	$1,500	$1,500	$1,500
SUPPLIES	$20,214	$21,023	$21,863	$22,738	$23,648
TRAVEL	$1,800	$1,800	$1,800	$1,800	$1,800
PATIENT CARE COSTS — INPATIENT					
PATIENT CARE COSTS — OUTPATIENT					
ALTERATIONS AND RENOVATIONS					
OTHER EXPENSES	$9,500	$9,880	$10,275	$10,686	$11,114
SUBTOTAL DIRECT COSTS	$175,428	$177,568	$184,538	$191,788	$199,328
CONSORTIUM/ CONTRACTUAL COSTS — DIRECT					
CONSORTIUM/ CONTRACTUAL COSTS — INDIRECT					
TOTAL DIRECT COSTS	$175,428	$177,568	$184,538	$191,788	$199,328

TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PERIOD OF SUPPORT *(Item 8a, Face Page)* → $ 928,650

JUSTIFICATION. Follow the budget justification instructions exactly. Use continuation pages as needed.

Personnel: The P.I. will devote 30% time/effort to supervise the overall project and the scientific endeavors of both the res. assoc., Katherine Sheldon (100% time/effort) and the res. tech., Christine Kearney (100% time/effort). The P.I. and res. assoc. will take responsibility for the chemical synthesis and evaluation of all transition state peptides, their conjugation to antigenic carriers, as well as production of monoclonal antibodies and their characterization.
They will

- design and synthesize several ELDKWA transition state gp41 peptides
- design and synthesize several new Aß transition state analog peptides
- carry out mass spectral analyses
- perform energy and conformational analyses of transition state vesus native peptides on graphics workstation
- plan and implement immunization protocols for each of the many hybridoma fusions required in this project
- perform hybridoma fusions to generate anti-transition state analog peptide antibodies
- use the comparative ELISA to screen the specificity of these new monoclonal antibodies
- develop and utilize catalytic antibody screening assays based upon hydrolytic cleavage of the ELDKWA peptide
- develop and utilize catalytic antibody screening assays based upon hydrolytic cleavage of Aß peptides
- purifiy antibodies to fully define their specificity and catalytic activity
- run kinetics analyses using the catalyic antibodies
- analyze the peptide cleavage products produced by the anti-Aß catalytic antibodies
- analyze the peptide cleavage products produced by the anti-gp41 catalytic antibodies
- carry out immunoblot analyses to identify and characterize the whole HIV cleavage products generated by the action of catalytic antibodies
- run partial N-terminal amino acid analysis on an automated Edman sequencer

A full time research technician is required to maintain the smooth operation of all of the support functions which allow the laboratory to run effectively. Some of the technical areas for this project include:

- collection of ascites from mice for monoclonal antibody isolation
- the purification and analysis of peptides by HPLC
- the analysis of peptide conjugates by PAGE
- determination of amino acid composition of the gp41 and Aβ transition state analog antigens
- maintenance of established cell lines and hybridoma clones
- immunization of animals
- bleeding mice to test for the production of antibodies
- running radioimmunoassays
- assisting in the performance of hybridoma fusions
- cryo-preservation of cells
- screening hybridoma clones by ELISA
- collection of ascites from mice for monoclonal antibody isolation
- purification and testing of monoclonal antibodies
- ordering and maintaining laboratory reagents and supplies
- sterilization of equipment, cages and surgical instruments
- preparation of buffers and reagents

Administrative assistance is needed for typing manuscripts and correspondence as well as for maintaining records of purchases and expenditures.

Equipment: A variable volume 12-channel pipetter is needed for removing, transferring and dispensing small aliquots of hybridoma supernatant from multiple 96-well plates during catalytic antibody screening procedures. The protein A Memsep and DEAE Memsep columns are needed to prepare highly purified monoclonal antibodies and Fab fragments which must be free of extraneous enzymatic activity. The liquid nitrogen freezer is requested because we have exceeded the storage capacity of our present freezer and we are producing new hybridomas at a prodigious rate.

Supplies: The experimental studies planned will require substantial supplies for peptide synthesis, hybridoma production and monoclonal antibody purification. The generation of numerous monoclonal antibodies is reflected in the need for purchasing adequate tissue culture supplies and for the considerable number of mice required for immunization and ascites production. The chemistry supplies are needed for the synthesis of each of the transition state peptides required for inducing and testing anti-transition state antibodies. The funds requested include the cost of F-moc-statine analogs (~$1,000/gm) that are required for several of the transition state peptides described in the proposal. Chromatography materials will be used during the synthesis of new peptides and for the purification of monoclonal antibodies.

Other Expenses: Animal housing is requested for maintenance of the mice used in the numerous hybridoma fusions which will be performed and for ascites production. Costs for publishing our findings are requested. Funds for travel to one or two national meetings per year are requested for both the P.I. and research associate to present research findings.

Duration of Support: Five years of support have been requested. I believe that the productivity and pioneering discoveries of my laboratory have proven our long-term commitment to the study of immunology and to the development of new strategies for realizing its clinical usefulness. The proposed research is fairly straightforward but many problems will have to be addressed to achieve our goals. The duration requested will allow us to follow up these novel developments and continue to gain new and important scientific insights. Moreover, the research has been designed so that its fundamental findings may have a direct application to human disease. I feel that the 5-year support is well justified for these reasons.

All categories are projected at a 4% increase per year. Equipment is projected at $1,500 for years 2-4 for the purchase of small equipment items to replace worn or obsolete instruments as needed. Fringe benefits are calculated at 29% for professional and non-professional personnel. Indirect costs are calculated at 92% based on salary and wage.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Photocopy this page or follow this format for each person

NAME	POSITION TITLE
Victor A. Raso	Sr. Scientist

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
Fordham University, Bronx, NY	B.S.	1967	Biology
Tufts University, Boston, MA	Ph.D.	1973	Biochemistry Immunochemistry

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. DO NOT EXCEED TWO PAGES.

Professional Experience:

1973-1974 Associate in Pharmacology, Harvard Medical School
1973-1981 Research Associate, Division of Biochemical Pharmacology, Dana-Farber Cancer Institute
1981-1988 Assistant Professor of Pathology, Dana-Farber Cancer Institute, Harvard Medical School
1988-1989 Principal Scientist, Boston Biomedical Res Inst., Dept. of Cell & Mol Biology
1989- Senior Scientist, BBRI, Dept. of Cell & Molecular Biology

Honors:

1983 Invited Lecturer and Tutor at the NATO Advanced Studies Inst., "Receptor Mediated Targeting of Drugs," Cape Sounion, Greece
1983 Lecturer, "Workshop on the Use of Monoclonal Antibodies in Tumor Therapy," National Institutes of Health, Bethesda, MD.
1984 Invited Lecturer at the American Association for the Advancement of Science Symposium on "Molecular Targeting of Drugs," NY, NY
1984 Invited Speaker at the Gordon Research Conference on "Drug Carriers in Biology and Medicine," Plymouth, NH
1986 Invited Lecturer at the Plenary Sess. in the UCLA Sympos. on Molecular and Cellular Biology, "Membrane-Mediated Cytotoxicity," Park City, UT
1986 Invited Speaker, Gordon Conference on "Drug Carriers in Biology and Medicine," Plymouth, NH
1987 Symposium Speaker, "Biological Approaches to the Controlled Delivery of Drugs: Barriers, Technology and Therapies," New York Acad. of Sciences, NY
1988 Session Chairman at the First Internat. Symp. on Immunotoxins, Durham, NC
1988 Recipient of the Pierce Immunotoxin Award
1990 Speaker at Second Internat. Sympos. on Immunotoxins, Buena Vista, FL.
1990 Chairman and Speaker, Fifth International Conference on Monoclonal Antibody Immunoconjugates for Cancer, San Diego, CA
1993 Invited to lecture at the American Association for the Advancement of Science Symposium "Drug Targeting in Diagnosis and Therapy," Boston
1996 Chairman and Speaker, Exploring and Exploiting Antibody and Ig Superfamily Combining Sites, Novel Immunotoxin Strategies, Keystone Symposia, Taos, NM

Publications (Partial):

1. Raso, V and Stollar, BD. Antibodies specific for conformationally distinct coenzyme-substrate transition state analogues. A fluorescence, N.M.R., circular dichroism and antibody study of N-(5-phosphopyridoxyl)-3'-amino-L-tyrosine. J. Amer. Chem. Soc. 1973; 95:1621.

2. Raso V and Stollar BD. The antibody-enzyme analogy. Characterization of antibodies to phosphopyridoxyltyrosine derivatives. Biochem. 1975; 14: 584-591.
3. Raso V and Stollar BD. The antibody-enzyme analogy. Comparison of enzymes and antibodies specific for phosphopyridoxyltyrosine. Biochem. 1975; 14: 591-599.
4. Raso V and Griffin T. Specific cytotoxicity of a human immunoglobulin directed Fab'-ricin A chain conjugate. J Immunol. 1980; 125 :2610.
5. Raso V and Griffin T. Hybrid antibodies with dual specificity for the delivery of ricin to immunoglobulin bearing target cells. Cancer Res. 1981; 41:2073.
6. Raso V, Ritz, J. Basala M and Schlossman SF. A monoclonal antibody-ricin A chain conjugate which is selectively cytotoxic for cells bearing the common acute lymphoblastic leukemia antigen (CALLA). Cancer Res. 1982; 42:457.
7. Raso V. Antibody mediated delivery of toxin molecules to antigen bearing target cells. In: Moller, G, Ed. Immunological Reviews: Antibody carriers of drugs and toxins in tumor therapy. Copenhagen: Munksgaard, 1982: 93-117.
8. Raso V and Basala M. A highly cytotoxic human transferrin-ricin A chain conjugate used to select receptor modified cells. J Biol Chem. 1984; 259:1143.
9. Raso V and Lawrence J. Carboxylic ionophores enhance the cytotoxic potency of ligand- and antibody-delivered ricin A chain. J Exp Med. 1984; 160:1234.
10. Raso, V, Watkins SC, Slayter H and Fehrmann C. Intracellular pathways of ricin A chain cytotoxins. In: Juliano R, Ed. Biological approaches to the controlled delivery of drugs: barriers, technologies and therapies. Ann. of the New York Acad. of Sci.. 1988; 507:172-185.
11. Raso V. and McGrath J. Diphtheria toxin cures athymic mice of human malignant mesothelioma. J. Natl. Cancer Inst. 1989; 81, 622-627.
12. Raso, V. The magic bullet - nearing the century mark. In: Osborn, M. ed. Seminars in Cancer Biology. Antibodies in diagnosis and therapy, 1990. vol. 1 pp 227-242
13. Recht, L., Torres, C. O., Smith, T. W., Raso, V. A., and Griffin, T. W. (1990) Transferrin receptor in normal and neoplastic brain tissue: Implications for brain-tumor immunotherapy. *J Neurosurg* **72**, 941-945.
14. Rakowicz-Szulczynska, E, Kaczmarski, W, Raso, V, Steimer, KS, Durda, P. Internalization of anti-gp120 monoclonal antibody and human antibodies by HIV-1-infected T lymphocytes. Antibody, Immunoconjugates, and Radiopharmaceuticals 1993; 6: 209-219
15. Griffin, T. and Raso, V. Monensin in lipid emulsion for the *in vivo* potentiation of ricin A chain immunotoxins. Cancer Res., 1991;51:4316-4322.
16 Griffin, T., Rybak, M.E., Recht, L., Singh, M., Salimi, A., Raso, V. Potentiation of antitumor immunotoxins by liposomal monensin. J. Natl. Cancer Inst. 1993; 85: 292-298.
17. Raso, V. Immunotargeting Intracellular Compartments. Anal. Biochem. 1994; 222: 294-304.
18. Griffin, T., Recht, L., Maher, E., Delichatsios, H., Raso, V. 1994; Antibody and Ligand-Toxin Conjugates as Therapeutic Agents. *In:* Cancer Therapy in the Twenty-First Century, Vol. I: Molecular and Immunologic Approaches (Huber, B.E. and Carr, B.I. eds). Futura Publishing Co.,
19. Recht, L., Raso, V. Davis, R. and Salmonsen, R. Immunotoxin sensitivity of CHO cells expressing human transferrin receptors with differing internalization rates. Cancer Immunol. Immunotherpy. 1996; 42: 357-361
20. Cunningham AL, Naif H, Saksena N, Lynch G, Raso V, Li S, Chang J, Alali M, Jozwiak R, et al.: HIV infection of macrophages and the pathogenesis of the AIDS dementia complex: Interaction of the host cell and viral genotype. Submitted. J. Leuk. Biol. 1998
21. Recht LD, Griffin TW, Raso V, Salimi AR. Potent cytoxicity of an antihuman transferrin receptor-ricin A-chain immunotoxin on human glioma cells *in vitro*. Cancer Res 1990; 50: 6696-6700.
22. Kelly M, Cunningham AL, Naif H, Adams SL, Lynch GW, Sloane A, Raso V: Dichotomous effects of ß-chemokines on HIV replication in monocytes and monocyte-derived-macrophages. (manuscript in preparation). 1998
23. Lynch GW, Sloane A, Raso V, Cunningham A: Direct evidence of CD4 oligomers in lymphoid and monocytoid cells. Submitted. Eur. J. Immunol. 1999
24. Raso, V. Intracellular targeting using bispecific antibodies. Meth. Mol. Med. 1998; in press.
25. Raso, V., Brown, M., McGrath, J., Liu, S. and Stafford, W. Antibodies capable of releasing diphtheria toxin in response to the low pH found in endosomes. J. Biol. Chem. 1997; 272: 27618-27622.
26. Raso, V., Brown, M. and McGrath, J. Intracellular targeting with low pH triggered bispecific antibodies. J. Biol. Chem. 1997; 272: 27623-27628.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Photocopy this page or follow this format for each person

NAME	POSITION TITLE
Katherine M. Sheldon	Research Associate

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Acadia University, Canada	BScH	1985	Chemistry
Univ. of Toronto, Ont., Canada	MSc	1987	Biochemistry
	PhD	1991	Biochemistry

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. DO NOT EXCEED TWO PAGES.

Professional Experience:

1991-1993 Postdoctoral fellow, Ontario Cancer Institute, Toronto, Ont., working on production of novel peptides that can be used as cellular targeting agents.

1993 - present Research Associate, Boston Biomedical Research Inst., Boston, MA. Immunochemistry and monoclonal antibody development

Publications:

Baumal, R., Law, J., Buick, R., Kahn, H., Sheldon, K., Colgan, T., and Marks, A. Monoclonal antibodies to an epithelial ovarian adenocarcinoma: Distinctive reactivity with xenografts of the original tumour and cultured cell line. 1986. *Cancer Res.***46**:3994-4000.

Bailey, D., Baumal, R., Law, J., Sheldon, K., Kannamphuzha, P., Stratis, M., Kahn, H. and Marks, A. Production of a monoclonal antibody specific for seminomas and dysgerminomas. 1986. *Proc. Natl. Acad. Sci. USA,* **83**:5291-5296.

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Sheldon, K., Marks, A., and Baumal, R. Sensitivity of multidrug resistant KB-C1 cells to an antibody-dextran-adriamycin conjugate. (1989) *Anticancer Res.* **9**:637-642.

Sheldon, K., Reilly, R., Baumal, R. and Marks, A. Imaging of human ovarian tumour xenografts in nude mice using a novel 111-In-labelled monoclonal antibody (1OB). (1991) *Nucl. Med. Biol.* **18:**519-526.

Zhang, A.M., Ballinger, J.R., Sheldon, K. and Boxen, I. Evaluation of reduction mediated labelling of antibodies with technetium-99m. (1992) *Appl. Radiat. Isot.* **19**: 607-609.

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Ballinger, J.R., Sheldon, K.M., Boxen, I., Erlichman, C., and Ling, V. Differences between accumulation of 99m-Tc-sestamibi and 201-Tl-thallous chloride in tumour cells: role of p-glycoprotein. (1995) *Quart. J. Nucl. Med. 39: 122-128.*

Remy, S., Reilly, R., Sheldon, K., and Gariepy, J. A new radioligand for the epidermal growth factor receptor: 111In labelled human epidermal growth factor derivatized with a bifunctional metal-chelating peptide. (1995) *Bioconj. Chem.* **6**: 683-690.

Sheldon, K., Liu, D., Ferguson, J., and Gariepy, J. Oligomers: design of *de novo* peptide-based intracellular vehicles. (1995) *Proc. Natl. Acad. Sci. USA* **92**: 2056-2060.

OTHER SUPPORT

Raso, V.
PENDING
NIH (Raso) 4/1/99-3/31/01
$70,000 30%

A Binary System for Cell-Targeted Delivery
The major goal of this project is to develop a two part system for delivering the isolated, non-toxic, complementary functional domains of a toxin to two distinct target sites on a cell. Improved targeting of cells and reduced non-specific toxicity will result because these non-toxic halves can combine to regain activity exclusively in the dual-labeled target cells.
OVERLAP
There is no scientific overlap between the application under consideration and this proposal which was submitted. There is budgetary overlap so if both are funded my percent effort would be adjusted and additional personnel would be recruited to fulfill the objectives.

Raso, V.
PENDING
Alzheimer's Association (Raso) 6/30/99-5/31/02
$60,000 20%

Vaccine To Modulate Systemic ß-Amyloid Levels
The major goal of this project is to develop a vaccine to induce conventional, systemic antibodies directed against the ß-amyloid peptide for potential use in the immunotherapy of Alzheimer's disease.
OVERLAP
There is minor scientific overlap between the application under consideration and this proposal which was submitted. There may be some budgetary overlap so if both are funded my percent effort would be adjusted appropriately in conjunction with agency staff.

Raso, V.
PENDING
NIH (Raso) 4/1/99- 3/31/02
$90,000 30%

Vaccine to Elicit Catalytic Anti-Cocaine Antibodies
The major goal of this project is to develop a vaccine which will induce catalytic antibodies capable of inactivating cocaine by hydrolytic cleavage.
OVERLAP
There is no scientific overlap between the application under consideration and this proposal which was submitted. There may be some budgetary overlap so if both are funded my percent effort would be adjusted appropriately in conjunction with agency staff.

Raso, V.
PENDING
NIH (Raso) 4/1/99- 3/31/01
$75,000 30%

Vaccine To Modulate Systemic ß-Amyloid Levels
The major goal of this project is to develop a vaccine to induce conventional, systemic antibodies directed against the ß-amyloid peptide for potential use in the immunotherapy of Alzheimer's disease.
OVERLAP
There is minor scientific overlap between the application under consideration and this proposal which was submitted. There may be some budgetary overlap so if both are funded my percent effort would be adjusted appropriately in conjunction with agency staff.

Raso, V.
PENDING
NIH (Raso) 7/1/99- 6/30/03
$198,885 50%
Cerebral Delivery of Vectorized Anti-Beta-Amyloid Antibody
The major goal of this project is to deliver anti-beta-amyloid antibodies into the brain so that they can delay or prevent the formation of cerebral plaques.
OVERLAP
There is no scientific overlap between the application under consideration and this proposal which was submitted. There may be some budgetary overlap so if both are funded my percent effort would be adjusted appropriately in conjunction with agency staff.

Raso, V.
PENDING
NIH (Raso) 7/1/99- 6/30/04
$198,254 50%
Catalytic Antibody Vaccine To Inactivate HIV
The major goal of this project is to develop catalytic antibody vaccines for the treatment and/or prevention of AIDS.
OVERLAP
There is complete scientific and budgetary overlap between the exploratory application under consideration and this long-term project which was submitted. Only one would be accepted if both are funded.

Raso, V.
PENDING
NIH (Raso) 12/1/99- 11/30/04
$175,885 30%
Immunotherapeutic Agents to Treat Alzheimer's Disease
The major goal of this project is to deliver anti-beta-amyloid antibodies into the brain so that they can delay or prevent the formation of cerebral plaques.
OVERLAP
There is some scientific overlap between the application under consideration and this proposal which was submitted. There may be some budgetary overlap so if both are funded my percent effort would be adjusted appropriately in conjunction with agency staff.

RESOURCES

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory: The PI occupies approximately 1000 sq. ft. of laboratory space on the 2nd floor of the Boston Biomedical Research Institute and has access to 900 sq. ft. of common space.

Clinical:

Animal: A complete small animal facility, including a room dedicated to breeding and handling of transgenic mice, is located in the basement of the Institute.

Computer: BBRI has a Microvax and we are connected to the Internet. Macintosh computers are also located in the lab and office for image processing, data analysis, literature searchs, sequence analysis, etc. The Silicon Graphics Iris Indigo XS24 graphics workstation at BBRI is available to this project for molecular modeling.

Office: Approximately 100 sq. ft. of office space is allotted to the PI on the second floor of the Boston Biomedical Research Institute.

Other: BBRI has a well equipped machine shop, an in-house library and access to the Tredwell library at the Massachusetts General Hospital. The BBRI has a PerSeptive Biosystems Voyager RP MALDI-TOF mass spectrometer. An in-house fluorescence flow cytometry facility is available. A molecular imager system is available for acquiring and digitizing radioisotopic images. The BBRI has an X-ray crystallography facility. We have access to an in-house Morphology Unit that provides Histology Services, Electron Microscopy Services and Confocal Microscopy Services.

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

Within the laboratory of the PI are: a scintillation counter, pH meter, semi-micro balance, top loading balance, Zeiss inverted tissue culture microscope, CO_2 incubator, fraction collectors, UV monitor, LKB spectrophotometer, microscope, clinical centrifuge, 6 ft. laminar flow hood, 1 liquid N_2 tank (full), flash evaporator, a multiple sample harvester, horizontal DNA/RNA gel electrophoresis apparatus, 2 microfuges, PAGE apparatus, a western blot apparatus, HPLC, and a video fluorescence microscope set-up.

Shared equipment at BBRI includes: a gamma counter, 3 scintillation counters, 2 autoclaves, 4 ultracentrifuges, a peptide sequencer, a warm room, 3 spectrophotometers, cold room, DNA synthesizer, a peptide synthesizer, 3 PCR machines, 4 HPLC units, a flow cytometry facility, EM 300 electron microscope, an AVIV circular dichroism spectrophotometer, a florescence lifetime apparatus, a DU 650 spectrophotometer, a phosphoimager apparatus, a MALDI-TOF mass spectrometer and an ELISA plate reader.

A. SPECIFIC AIMS

Existing paradigms for antibody and vaccine development have not led to the efficacious treatment or prevention of AIDS or Alzheimer's disease. Innovative new approaches are therefore needed to drive the field of therapeutic immunology foward so that it can accommodate the special problems posed by such diseases. The long-term objective of this project is to test a novel concept in antibody design. We will develop specific transition state analog antigens that elicit catalytic antibodies with a capacity to proteolytically cleave the target protein. Thus, more effective and prolonged therapeutic action should be achieved by using catalytic versus ordinary antibodies.

Central to testing this hypothesis and a major goal of the proposed research is the generation of exceptional catalytic antibodies which cleave the peptide bond. Obtaining bona fide catalytic antibodies from transition state vaccines is not a trivial task and will require a long-term commitment. However, several novel transition state analog gp41 and Aß peptides have already been produced and some very interesting and potentially catalytic antibodies have been obtained. Different transition state peptide analogs will be tested and many fusions are planned to optimize our chances for obtaining the best possible reagents. Screening those antibodies for catalytic activity and examining some select examples in depth represents the most immediate aim of this research. Our rational assembly of reagents and techniques currently puts us in a unique position to establish the scientific basis for this novel and potentially high impact approach to treating disease.

A.1 Produce Statine Transition State Peptide Analogs of HIV gp41 and Aß

a. Synthesize an elongated gp41 peptide containing a tetrahedral statine residue at a critical position in the conserved gp41 ELDKWA viral neutralization epitope.

b. Synthesize elongated peptides having tetrahedral statine analogs at critical positions in the sequence of the Alzheimer's disease associated Aß 43-mer.

A.2 Produce Reduced Peptide Bond Transition State Analogs of HIV gp41 and Aß

a. Synthesize elongated gp41 peptides containing a tetrahedral reduced peptide bond at critical positions in the conserved gp41 ELDKWA viral neutralization epitope.

b. Synthesize elongated peptides having tetrahedral reduced peptide bond at critical positions in the sequence of the Alzheimer's disease associated Aß 43-mer.

A.3 Produce Phosphonate or Phosphonamidate Analogs of HIV gp41 and Aß

a. Synthesize elongated gp41 peptides containing a phosphonate or phosphonamidate bond at critical positions in the conserved gp41 ELDKWA viral neutralization epitope.

b. Synthesize elongated peptides containing a phosphonate or phosphonamidate moiety at critical positions in the Alzheimer's disease associated Aß 43-mer.

A.4 Elicit Monoclonal Antibodies with the Novel Transition State Analog Peptides

a. Immunize mice with immunogens produced by coupling the different gp41 ELDKWA and Aß transition state analog peptides to an antigenic carrier protein.

b. Establish antibody-producing hybridoma clones and characterize the anti-peptide specificity of these antibodies using a comparative ELISA.

c. Screen hybridoma supernatants using proteolytic assays based upon the cleavage of soluble ^{125}I-peptide, fluorogenic peptide or solid phase ^{125}I-peptide substrates.

d. Induce ascites production and isolate monoclonal antibodies that show either catalytic activity or a high preference for binding the transition state peptide.

e. Characterize further the binding specificity, the catalytic activity, as well as the gp41 and Aß cleavage products generated by the antibodies.

B. BACKGROUND and SIGNIFICANCE

B.1 Application of Proteolytic Catalytic Antibodies to Disease: More than 20 years ago, Raso and Stollar published the first formal study expressly aimed at inducing antibodies possessing catalytic activity (1-3). A transition state enzyme inhibitor was designed, synthesized and used as a hapten to elicit complementary antibody combining sites that would mimic the chosen enzyme active site. A fivefold rate enhancement was achieved for the tyrosine transamination reaction occurring at these antibody sites versus free in solution. This modest acceleration is actually quite significant, considering that this result was obtained well before development of hybridoma technology, so only heterogeneous populations of affinity purified rabbit serum antibodies could be used.

With the emergence of monoclonal antibody techniques, the field of catalytic antibodies has exploded, largely due to recent efforts from the laboratories of Lerner, Benkovic and Schultz (4). Homogeneous catalytic antibodies can now be selected, purified and studied in the absence of any competing non-catalytic species. Numerous catalytic antibodies, accelerating a large array of diverse chemical reactions, have been produced within the last several years (4-10). In light of the rapid progress since our early pioneering work, it is apparent that the time is now ripe to apply this unique technology to the pressing health problems confronting medical scientists.

Having this end in mind, we have synthesized several novel antigens which are designed to elicit catalytic antibodies that irreversibly inactivate disease causing entities. These therapeutic antibodies would function by specifically cleaving a critical peptide bond in the protein structure of viruses, bacterial toxins, enzymes or disease causing polypeptides within the body. Two eminently relevant targets have been selected to develop the fundamental concepts, techniques and reagents necessary for testing the potential of this general therapeutic approach.

We chose to target the conserved ELDKWA sequence which has been identified as a vital neutralization epitope located within the integral gp41 transmembrane protein of the HIV envelope (11, 12). Catalytic antibodies, by permanently modifying the viral envelope, should attenuate or abolish HIV infectivity and thereby impede the progression of AIDS. The other molecule that we decided to target is the 43 amino acid residue beta-amyloid peptide (Aß) which is central to Alzheimer's disease pathogenesis. This full-length peptide deposits as amyloid plaques in the brain (13) and those lesions most likely lead to neurological damage and the symptoms of Alzheimer's disease. Given the importance of intact Aß to plaque formation, any preemptive cleavage of this peptide by a catalytic antibody should yield harmless, amyloidogenically inactive fragments of Aß.

Since they exhibit turnover, each catalytic antibody will continuously inactivate many target molecules rather than just binding stoichiometrically. Thus, compared to ordinary neutralizing antibodies, catalyic antibodies would offer a tremendous boost in therapeutic efficacy.

B.2 Novel Transition State Peptide Analogs: To create stereochemically altered peptides we inserted statine, reduced peptide bond and phosphonate or phosphonamidate moieties into the gp41 ELDKWA and Aß amino acid sequences. These transition state analog molecules have tetrahedral geometry and mimic the peptide bond during hydrolysis. Thus those modified peptides will constitute the basis of our antigens for inducing catalytic antibodies that specifically cleave gp41 or Aß.

Enzymatic hydrolysis of peptide bonds involves nucleophilic attack on the carbonyl group aided by general base catalysis of proton removal from the attacking nucleophile and eletrophilic assistance to increase polarization of the -C-O bond. Breakdown of the tetrahedral intermediate formed is aided by general acid catalysis to assist departure of the amine. Uncatalyzed rates of peptide hydrolysis in water, $3 \times 10^{-9}\ s^{-1}$, predict a half-life of 7 years (14). Proteases can impart a rate enhancement of 10^9, reducing this half-life to < 1 second (15). A catalytic antibody that accelerates cleavage 2,500-25,000-fold would put the half-life for hydrolysis in a measurable range of 2-20 hours.

Statine and reduced peptide bond analogs have not been previously used to elicit proteolytic antibodies. The "statyl" moiety is derived from naturally evolved protease transition state inhibitors like amastatin, pepstatin and bestatin. Statine-based inhibitors effectively block the activity of aminopeptidases, aspartic proteases and the HIV protease. Peptides containing a statine residue offer some novel features for the induction of catalytic antibodies. The statyl moiety has tetrahedral bond geometry, its length is extended by two CH_2 units, there is a strategically placed OH group and the

structure has no charge. Statine-based analogs should elicit a class of antibodies which is significantly different from that obtained by employing the commonly used phosphonate analogs.

Reduced peptide bond analogs also introduce a tetrahedral configuration but do not increase the distance between amino acid residues. A positively charged secondary amine replaces the amide nitrogen and should elicit a complementary negatively charged side chain at a proximal locus in the antibody combining site. Such ancillary glutamyl or aspartyl groups could assist antibody-mediated catalysis of peptide cleavage via acid-base exchange.

Phosphonate and phosphonamidate derived oligopeptides are extremely potent inhibitors of zinc metalloproteases, thermolysin and carboxypeptidases, in some cases displaying K_i values in the femtomolar range (16-18). Pepstatin and synthetic peptide derivatives based upon its unusual amino acid, statine, are potent inhibitors of aspartic proteases such as porcine pepsin (K_D = 45 pM) (19), penicillopepsin (K_i = 100 pM) (20) and HIV-1 protease (K_i = 18 nM) (21). Crystallographic analyses verify that phosphonate and statyl peptides bind to their respective enzyme active sites in a manner consistent with that of a good mimic of the tetrahedral transition state intermediate (20, 22).

Transition state haptens containing phosphonate or phosphonamidate moieties have been used to induce a diverse array of catalytic antibodies (8, 9, 23-27). These antibodies accelerated ester and amide hydrolysis reactions by a factor of 500-260,000-fold over the uncatalyzed reaction rate. Sequence-specific peptide cleavage at a 10^5-fold enhanced rate has been achieved using antibodies elicited by a Co(III) trien-peptide hapten (10). The mechanism may involve stabilization of the transition state relative to the substrate and product (28). Catalysis can also be due to active site residues that participate in the reaction via a covalent acyl-antibody intermediate (25).

B.3 Extended Sequence Transition State Analog Peptides: Despite its sound theoretical rationale, the prior use of phosphonate analogs for producing catalytic antibodies with proteolytic activity, as opposed to esterolytic or amideolytic activity, has not been fruitful. One possible reason for previous difficulties in obtaining proteolytic catalytic antibodies is the small size and artificial structure of the transition state analogs used as immunogens. We believe each of our transition state peptide analogs have an improved chance for inducing proteolytic antibodies due to their greater chain length and the use of native amino acid residues adjacent to the modified peptide bond. Our approach takes into account the subsite nature of many proteolytic enzymes.

We chose to use long transition state peptides because of two important considerations. An extended sequence is required to ensure that the predetermined cleavage specificity of the resulting catalytic antibody will exclusively recognize the ELDKWA or Aß epitope. However, examination of naturally occurring proteases suggest that chain length can also play an important role in the catalytic mechanism. X-ray crystallography studies provide structural evidence for multiple enzyme-peptide interactions along elongated groove active sites. There are many examples (29-32) where extension of the peptide substrate greatly increases the k_{cat}/K_m. A comparison of the cleavage of Ac-Ala-OCH_3 versus Ac-Ala-Ala-Ala-OCH_3 by elastase or subtilisin shows that the k_{cat}/K_m is 2000-4000 times higher for the longer substrate. Similarly, the k_{cat}/K_m is 2000 times higher for pepsin cleavage of the Phe-Phe bond in Z-Ala-Ala-Phe-Phe-O-4-pyridylpropyl than for its cleavage in the shorter Z-Phe-Phe-O-4-pyridylpropyl substrate. By analogy we designed our long transition state peptide analogs to induce complementary elongated antibody combining sites which mimic proteolyic enzyme active site clefts. Such extended grooves with multiple contact areas between the antibody and peptide may be more effective for inducing strain in the peptide bond, which should promote its hydrolytic cleavage.

While it was anticipated from the start that generating catalytic antibodies would not be an easy task, we have been pleasantly surprised that the initial use of our antigens has elicited numerous bona fide anti-transition state analog antibodies. We also realize that demonstrating unequivocally that some of these antibodies or those from the planned subsequent fusions have catalytic activity remains a paramount goal of this project.

An advantage to choosing the ELDKWA epitope and Aß as targets for developing catalytic antibodies is that carrier-bound, small peptides readily elicit specific monoclonal antibodies that react with the intact native structure (33-37). Antibody binding to whole HIV or Aß can occur when the virus is attached to cells and when Aß is incorporated into amyloid plaques. A large array of HIV and Aß peptide antigens have been used to generate antibodies (33-37).

B.4 HIV Envelope Target Sites: Targeting our catalytic antibodies to the best possible site on HIV is an important consideration for their eventual usefulness as therapeutic agents. We have used several criteria to help design antibodies that afford the greatest likelihood for impacting AIDS. We chose to target known neutralization determinates on the virus because those epitopes are immunogenic, functionally significant and readily accessible to the binding of antibodies. A linear determinant rather than discontinuous conformational determinant is required since we need to construct a synthetic transition state analog for immunization. Sites which are relatively conserved or have single amino acid variants are preferred so that our reagents will be broadly applicable. Finally, to be relevant, the neutralization epitope must be expressed on a high percentage of primary clinical isolates and not restricted to laboratory stocks of HIV.

Fortunately, a recent evaluation of the neutralization activity of various monoclonal antibodies on primary isolates (12) has identified a gp41 site that meets the criteria that we have established. Accordingly, the gp41 ELDKWA epitope as defined by the broadly neutralizing 2F5 human monoclonal antibody (11) has become the prime focus of our research. An important feature of this ELDKWA sequence is its accessibility on the oligomeric form of the envelope glycoproteins (38, 39). Therefore, we are currently developing transiton state peptide vaccines designed to elicit catalytic antibodies that will cleave at the critical ELDKWA locus and inactivate HIV.

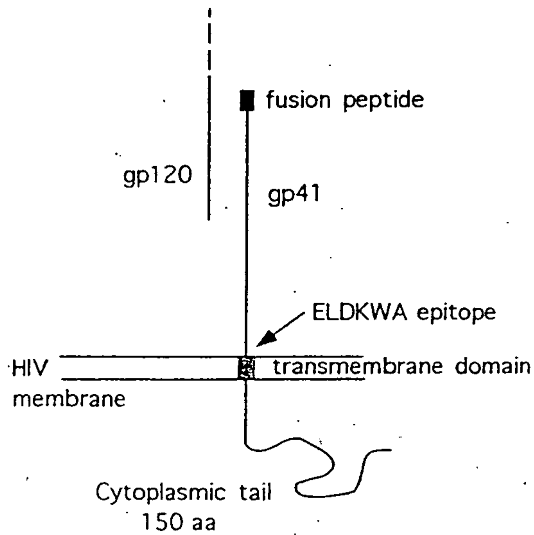

Fig. 1 ELDKWA Site on gp41

The 2F5 antibody was derived from a person infected with the clade B isolate and it neutralized 7 of the 9 clade B isolates tested (12). This antibody was equally effective against clade A-F virus isolates (40). Susceptible isolates had either the ELDKWA sequence or a close homolog sequence (ALDKWA and ALDKWQ). Only core changes like D→G or K→T precluded both binding and neutralization by 2F5 (12, 41). Thus, by targeting this site for modification using catalytic antibodies we hope to minimize the chance for natural selection of resistant HIV varients which might escape treatment. In the event that they are needed, we can easily synthesize new transition state analog peptides resembling point mutated variants which appear due to the presence of antibody during treatment. The ELDKWA site is located immediately above the gp41 ectodomain/HIV membrane junction (Fig. 1) (38, 39, 42). Given the importance of this gp41 neutralization region to viral function (38, 43), any premature or preemptive cleavage at that key site by a catalytic antibody could release the entire ectodomain and yield an inactive HIV-1 particle. Thus the spread of AIDS throughout the body would be impeded by attenuating or abolishing viral infectivity.

When we initially envisioned this catalytic antibody approach the GPGRAF principal neutralization determinant located at the V3 loop of gp120 appeared to be a reasonable choice as a target for antibody-mediated cleavage. Unfortunately, even though anti-V3 antibodies bind to and effectively neutralize laboratory isolates like HIV_{MN} they do not consistently neutralize primary isolates (12). Substantial preliminary data has already been obtained regarding this V3 target site. Therefore some of that work is presented in partial support of this proposal. The underlying strategy, transition state analog antigen design and monoclonal antibody methodology are analogous regardless of whether the target epitope is ELDKWA on gp41 or GPGRAF on the gp120 V3 loop.

B.5 The Importance of the Beta-Amyloid Peptide: Alzheimer's disease is a progressive and ultimately fatal form of dementia that affects a substantial portion of the elderly population. Definitive diagnosis at autopsy relies on the presence of neuropathological brain lesions marked by a high density of amyloid plaques. The principal component of these plaques is a 39-43 residue beta-amyloid peptide (Aß). Each plaque contains ~ 20 fmole (80 picograms) of this 4 kDa peptide (44).

There is ample evidence to support the pathogenic role of Aß in Alzheimer's disease. For example, patients with Down's syndrome have an extra copy of the beta-amyloid precursor protein gene due to trisomy of chromosome 21 (45, 46). They correspondingly develop an early-onset Alzheimer's disease neuropathology at 30-40 years of age. Moreover, early-onset familial Alzheimer's

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disease can result from mutations in the beta-amyloid precursor protein gene which fall within or adjacent to the Aß sequence (47). These observations are consistent with the notion that deposition of Aß as plaques in the brain might be accelerated by elevation of its extracellular concentration (48).

Observations have indicated that amyloid plaque formation may proceed by a crystallization type mechanism (49, 50). According to this model, the seed that initiates plaque nucleation is an Aß which is 42 or 43 amino acids long ($Aß_{1-43}$). One might expect that Aß plaque formation in the brain is an irreversible process with little chance for therapeutic intervention. However, important experiments suggest that a dynamic equilibrium may exist between soluble Aß and fibrillar Aß deposited as plaques in the brain (51-55). Those results provide hope for using catalytic antibodies or their small fragments as a gentle means for eradicating plaques in Alzheimer's patients.

Given the central role played by Aß in Alzheimer's disease, it has become increasingly important to understand the interrelationship between the different pools of these molecules in the body. Free Aß is present in the blood arises from cells in the peripheral tissues while Aß species found in the central nervous system are derived from peptide released from brain cells. However, since the peptides are identical regardless of origin, intercommunication between the brain/cerebrospinal fluid pool and the blood/tissue pool is a distinct possibility. The preceding observations indicate that the onset of plaque formation, plaque size or the number of plaques might be influenced by reducing levels of soluble Aß in the brain and these may in turn be linked to Aß levels in the blood. Catalytic anti-Aß antibodies would provide a highly efficient and permanent means of depleting these peptides in the blood. In the presence of catalytic antibodies the circulating Aß would be continuously degraded into harmless fragments (Fig. 2). Aß equilibria would be perturbed by gradually depleting soluble Aß in the blood stream and this could correspondingly reduce Aß levels in the brain (Fig. 2). Systemic catalytic antibodies would also prevent any passage of intact Aß from the blood into the CNS. Direct infusion of catalytic antibodies into the central nervous system is also feasible but a more elegant vector-mediated method for delivering blood-borne antibodies across the blood-brain barrier has been devised (56-59).

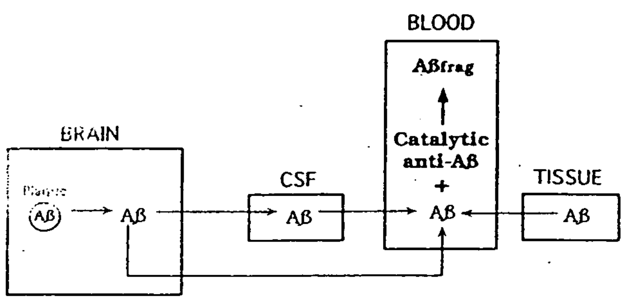


Fig. 2 Equilibria Altered by Catalytic Anti-Aß

B.6 Summary: The proposed research explores an entirely new conceptual framework for therapeutic antibody and vaccine development. It will allow us to identify, produce and characterize several new transition state antigens designed to elicit catalytic antibodies which selectively cleave target proteins at any specific sequence site. Locus-specific cleavage on the level of proteins would be comparable in importance to the use of restriction enzymes on the level of DNA.

While it has proven difficult to induce catalytic antibodies that will sever a peptide bond, we have introduced several new ideas which should breathe new life into that pursuit. Our transition state analogs have been designed to be elongate peptides which incorporate a unique tetrahedral bond at the proposed cleavage site. This use of an extended amino acid sequence and of several subtly different transition state mimics should promote the induction of catalytic antibodies.

To test our ideas, we have selected two relevant disease causing entities, the HIV gp41 envelope protein and the Alzheimer's disease associated Aß peptide. Our hypothesis predicts that antibodies with continuous catalytic activity should show a significantly boosted therapeutic effect. They would neutralize and then permanently inactivate these targets, rather than just transiently binding to them. We will directly compare the action of the new catalytic antibodies with conventional neutralizing antibodies on the functional activities or either HIV or Aß. Those antibodies and transition state antigens will then be refined to achieve our longer term objective, the production of clinically useful antibodies and vaccines for the treatment or prevention of AIDS and Alzheimer's disease.

C. PRELIMINARY STUDIES

C.1 Produce Statine Transition State Peptide Analogs of HIV gp41 and Aß

a. Statine based gp 41 transition state analog peptide: A statine (Sta) transition state peptide analog of the gp41 neutralization epitope, ELDKWA, has been synthesized in this laboratory (Fig. 3). Replacement of the proposed scissile peptide linkage between Leu_{663} and Asp_{664} (↓) with a "statyl" (*-CHOH-CH₂-CO-NH-*) moiety is designed to elicit catalytic antibodies that will hydrolytically cleave the virus at this site in the gp41 region of the envelope. Three additional amino acids in the gp41 sequence were included as a spacer and a Cys residue was placed at the position of Glu_{559} to provide a suitable linkage group for coupling this transition state peptide (Cys-Leu-Leu-Glu-***Sta***-Asp-Lys-Trp-Ala-Ser) to an antigenic carrier protein (Fig. 3).

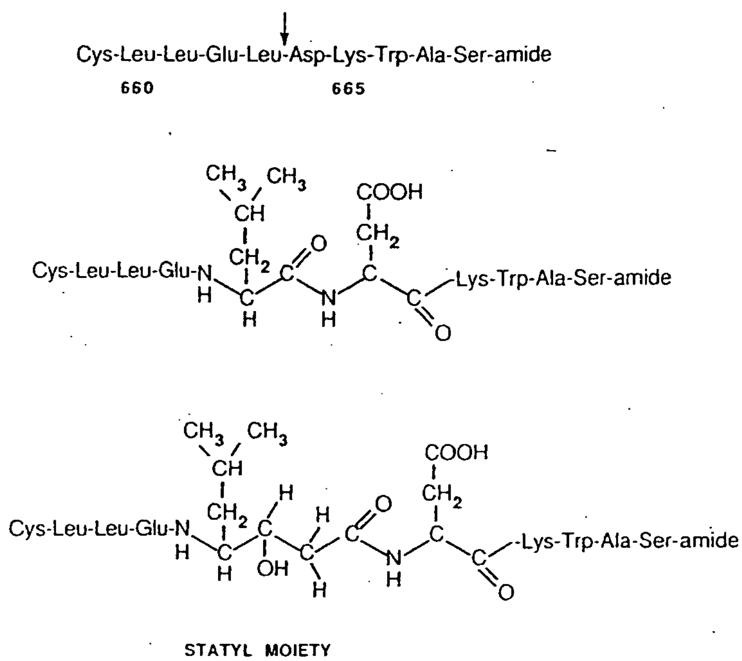


Fig. 3 Comparison of the gp41 Neutralization Epitope and the Statine Transition State Analog

The statine transition state analog peptide was synthesized using automated Fmoc chemisty. This was feasible due to the commercial availability of Fmoc-statine, [N-Fmoc-(3S,4S)-4-amino-3-hydroxy-6-methylheptanoic acid]. The peptide was purified by HPLC and its composition verified by its mass spectrum and amino acid analysis. A MALDI-TOF mass spectrograph of our Cys-terminated statine transition state gp41 peptide is shown in Fig. 4; [calcd 1,222.5 (M+1), found 12,223.7].

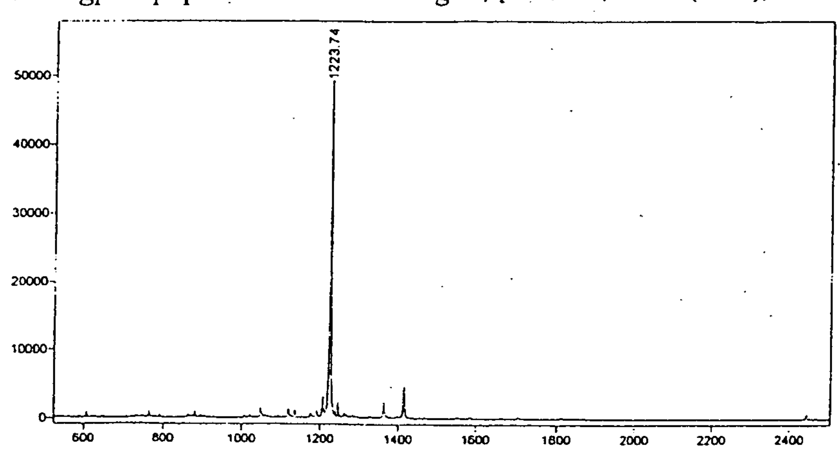
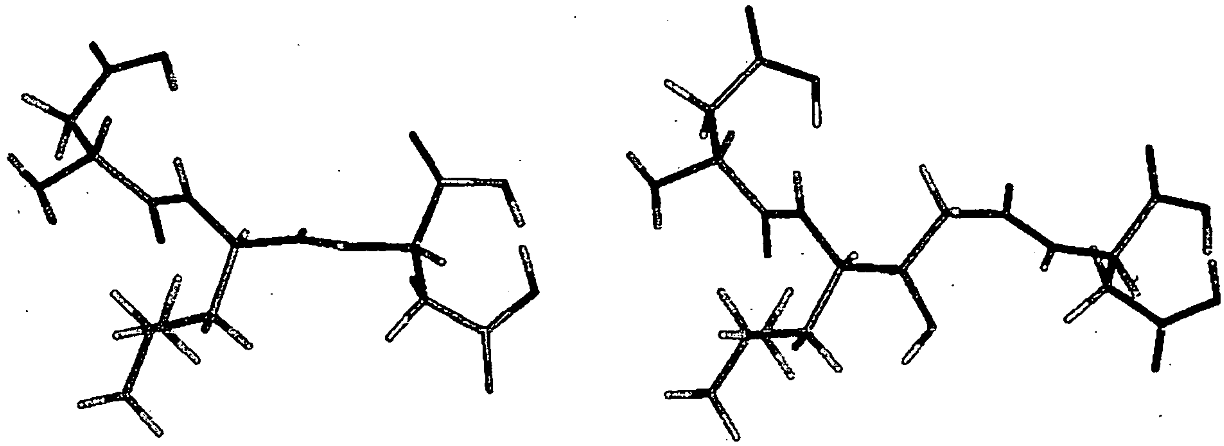


Fig. 4 Mass Spectrograph of the Cys-Leu-Leu-Glu-*Sta*-Asp-Lys-Trp-Ala-Ser Transition State Peptide

A structural comparison was made between the native peptide and the statine transition state peptide using a graphics workstation (Fig. 5). An energy minimization algorithm (1000 iterations) was applied to the peptides to arrange each in its most favorable conformation.



↑ HO ↑

Fig. 5 -Glu-Leu---CO-NH—Asp- -Glu-Leu-CH-CH_2-CO-NH-Asp-

The peptide link ***-CO-NH-*** (↑) between Leu_{663} and Asp_{664} of the gp41 epitope peptide was replaced with an elongated "statyl" moiety ***-CHOH-CH_2-CO-NH-*** (↑). The orientation shown above illustrates the difference between a planar peptide link of the natural peptide on the left versus an extended, tetrahedral "statyl" moiety in the transition state peptide on the right.

We determined if the novel statine peptide could be sterically accommodated by the combining site of the 2F5 neutralizing antibody that was generated against native gp41 ELDKWA epitopes by an AIDS patient. An ELISA was set-up using either the Cys-Leu-Leu-Glu-***Sta***-Asp-Lys-Trp-Ala-Ser statine peptide, two different overlapping gp41 peptides containing the native ELDKWA sequence or an adjacent gp41 peptide without that sequence as a negative control (Table I). Our statine peptide (Fig. 5) did cross-react with this 2F5 antibody suggesting that the stereochemically altered analog can be forced to conform to a combining site that is complementary with the naturally occurring epitope. We, of course, will be generating monoclonal antibodies that are designed to accomplish the opposite goal; namely, constrain the native ELDKWA epitope into a transition state-like configuration.

Table I **ELISA for Binding of 2F5 to the Statine and Native gp41 Peptides**

	Antibody Bound (O.D. 450nm)		
Peptide on Plate	**No Antibody**	**2F5 (10^{-9}M)**	**2F5 (10^{-8}M)**
No Peptide	0.016	0.054	0.426
Control Peptide	0.017	0.075	0.399
Statine Peptide	0.030	0.453	1.471
Native Peptide 1	0.024	0.552	1.522
Native Peptide 2	0.019	0.422	1.868

Our statine-based ELDKWA transition state peptide analog peptide has been thioether-linked to maleimide activated keyhole limpet hemocyanin (KLH). The antigen was emulsified in complete Freunds adjuvant and injected i.p. into mice. This new immunogen is designed to induce anti-transition state peptide antibodies that will recognize and potentially cleave the gp41 ELDKWA neutralization epitope.

A phenylalanine statine transition state analog encompassing the crown region of the V3 loop of gp120 on the HIV envelope (Lys-Arg-Ile-His-Ile-Gly-Pro-Gly-Arg-Ala-Phe-Tyr-Thr-Thr-Lys) has previously been synthesized in this laboratory. Replacement of the proposed scissile peptide linkage between Phe_{324} and Tyr_{325} (↓) with a "statyl" moiety (***-CHOH-CH_2-CO-NH-***) is designed to elicit

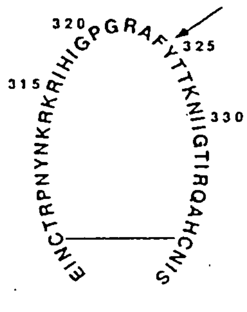


Fig. 6

catalytic antibodies that will hydrolytically cleave the virus at this site in the V3 loop (Fig. 6). A Cys residue was placed at the position of Arg_{315} to provide a suitable linkage group for coupling this (Cys-Lys-Arg-Ile-His-Ile-Gly-Pro-Gly-Arg-Ala-***PhSta***-Tyr-Thr-Thr-Lys) peptide to an antigenic carrier protein.

The phenylalanine statine transition state peptide was synthesized using automated Fmoc chemisty. This was feasible due to the recent availability of Fmoc-"phenylalanine statine" (***PhSta***), [N-Fmoc-(3S,4S)-4-amino-3-hydroxy-5-phenylpentanoic acid] from a commercial source. The peptide was tested for purity by HPLC and its composition was verified by mass spectral and amino acid analysis.

b. Statine based Aß transition state analog peptides: The amino acid sequence of the 43 residue beta-amyloid peptide (Aß) is shown in Fig. 7. Predicting precisely which site on the Aß peptide will be ultimately best suited for antibody-mediated therapy is difficult. Therefore key epitopes on

H_2N-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIAT-CO_2H

1 10 20 30 40

Fig. 7

the Aß 43-mer were chosen as targets for the catalytic antibodies. The resulting panel of monoclonal antibodies will be screened *in vitro* to identify those having catalytic activity.

A series of statine transition state analogs encompassing the carboxy-terminal region of Aß (Cys-Met-Val-Gly-Gly-Val/**Sta**-Val/**Sta**-Ile/**Sta**-Ala-Thr) has been synthesized (Fig. 8).

↓ ↓ ↓

Met-Val-Gly-Gly-Val-Val-Ile-Ala-Thr-CO_2H

35 40

Fig. 8

Replacement of the proposed scissile peptide linkage between Val_{39} and Val_{40} (↓), Val_{40} and Ile_{41} (↓) and Ile_{41} and Ala_{42} (↓) with a "statyl" moiety (-CHOH-CH_2-CO-NH-) is designed to elicit catalytic antibodies that will hydrolytically cleave Aß at one of these sites (Fig. 8). A Cys residue was placed at the position of Leu_{34} (Fig. 7) to provide a suitable linkage group for coupling this peptide to a maleimide-activated carrier protein.

A series of phenylalanine statine (**PhSta**) transition state analogs encompassing the central region of Aß (Cys-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe/**PhSta**-Phe/**PhSta**-Ala-Glu-Asp-Val-Gly-amide) was synthesized in this laboratory (Fig. 9).

↓ ↓

Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-amide

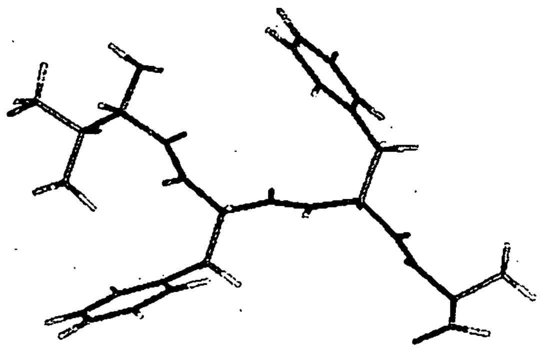
10 15 20 25

Fig. 9

Replacement of the proposed scissile peptide linkage between Phe_{19} and Phe_{20} (↓) and Phe_{20} and Ala_{21}(↓) with a statyl moiety (**-CHOH-CH_2-CO-NH-**) is designed to elicit catalytic antibodies that will hydrolytically cleave Aß at these sites (Fig. 9). A Cys residue was placed at the position of Gly_9 (Fig. 7) to provide a sulfhydryl linkage group for coupling the peptide to antigenic, maleimide-activated carrier proteins such as KLH.

The statine and phenylalanine statine transition state peptides were synthesized using automated Fmoc chemisty. We used commercially available Fmoc-statine (**Sta**), [N-Fmoc-(3S,4S)-4-amino-3-hydroxy-6-methyl heptanoic acid] and Fmoc-"phenylalanine statine" (**PhSta**), [N-Fmoc-(3S,4S)-4-amino-3-hydroxy-5-phenylpentanoic acid]. Each peptide was tested for purity by HPLC and its composition was verified by mass spectral and amino acid analysis.

A structural comparison (Fig. 10) was made between the native Aß peptide and the transition state phenylalanine statine Aß peptide using a graphics workstation. An energy minimization algorithm (2000 iterations) was applied to arrange each peptide in its most favorable conformation.



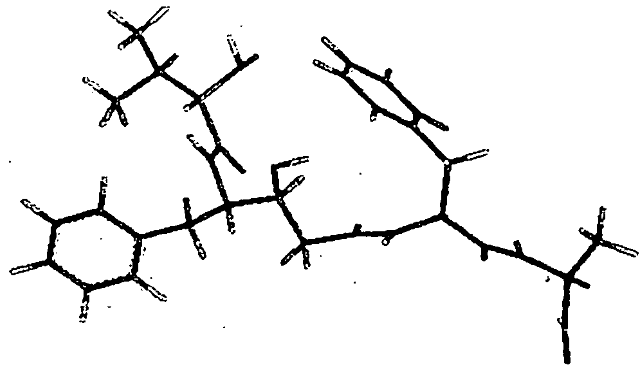


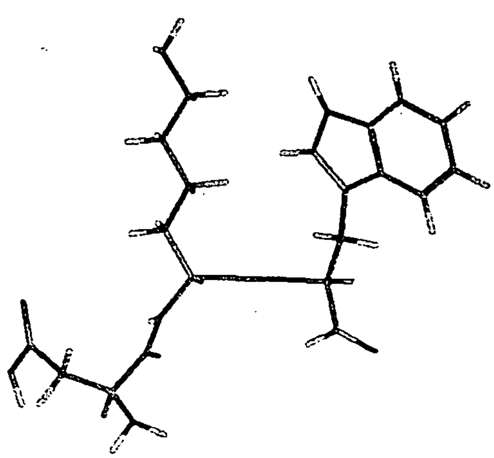
Fig. 10 Val-Phe-CO-NH-Phe-Ala ↑ HO Val-Phe-CH-CH$_2$-CO-NH-Phe-Ala ↑

The peptide link **-CO-NH-** (↑) between Phe$_{19}$ and Phe$_{20}$ was replaced with an elongated "statyl" moiety **-CHOH-CH$_2$-CO-NH-** (↑) and an energy minimization was applied. This orientation shows the difference between the planar peptide link **-CO-NH-** (↑) of natural Aß (left) versus the extended, tetrahedral "statyl" moiety **-CHOH-CH$_2$-CO-NH-** (↑) in the transition state peptide (right).

An antibody combining site complementary to a tetrahedral statine transition state analog will force the planar peptide bond of the Aß substrate into a transition state-like conformation. Such distortion should catalyze the cleavage of Aß at that locus in the peptide sequence.

C.2 Produce Reduced Peptide Bond Transition State Analogs of HIV gp41 and Aß

a. Reduced peptide bond based gp 41 transition state analogs: A reduced peptide bond linkage can be easily placed at any site in the gp 41 ELDKWA epitope sequence. The first reduced peptide bond transition state analog of ELDKWA that we synthesized is Leu-Glu-Leu-Asp-Lys-**CH$_2$-NH$_2$$^+$**-Trp-Ala-Ser-Leu-Cys-amide; [calcd 1,164 (M+1), found 1,164]. Automated Fmoc chemistry was used to begin synthesis of the blocked, resin-linked Trp-Ala-Ser-Leu-Cys-amide. A pre-synthesized Fmoc-Lys aldehyde was then added manually and after the imide was reduced, automated synthesis was resumed to complete the peptide (60).



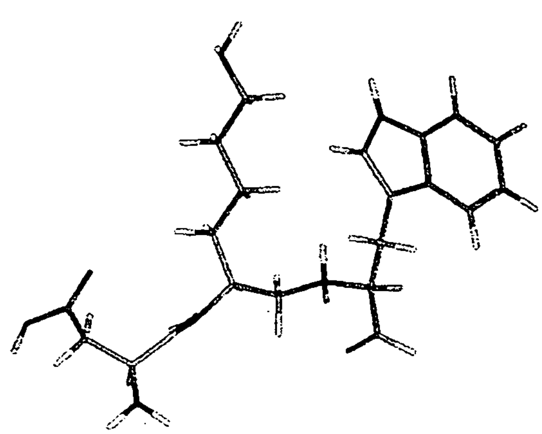


Fig. 11 ↑ Asp-Lys-CO-NH-Trp ↑ Asp-Lys-CH$_2$-NH$_2$$^+$-Trp

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A structural comparison (Fig. 11) was made between the native ELDKWA peptide and the reduced peptide bond transition state analog using a graphics workstation. An energy minimization algorithm (2000 iterations) was applied to arrange each peptide in its most favorable conformation.

The peptide link **-CO-NH-** (↑) between Lys_{672} and Trp_{673} of the gp41 epitope peptide was replaced with a reduced peptide bond **-CH_2-NH_2^+-** (↑) and an energy minimization was applied. This orientation shows the difference between the planar peptide link **-CO-NH-** (↑) of natural Aß (left) versus the corresponding tetrahedral moiety **-CH_2-NH_2^+-** (↑) in the reduced peptide bond transition state analog (right).

We determined if this novel reduced peptide bond analog could be sterically accommodated into the combining site of the 2F5 neutralizing antibody that was generated against native gp41 ELDKWA epitopes by an AIDS patient. An ELISA was set-up using either the Leu-Glu-Leu-Asp-Lys-**CH_2-NH_2^+**-Trp-Ala-Ser-Leu-Cys-amide reduced peptide bond analog, two different overlapping gp41 peptides containing the native ELDKWA sequence or an adjacent gp41 peptide without that sequence as a negative control (Table II). Our reduced peptide bond analog (Fig. 11) did cross-react with this 2F5 antibody suggesting that the stereochemically altered analog can be forced to conform to a combining site that is complementary with the naturally occurring epitope. We, of course, will be generating monoclonal antibodies that are designed to accomplish the opposite goal; namely, constrain the native ELDKWA epitope into a transition state-like configuration.

Table II **ELISA for Binding of 2F5 to the Reduced and Native gp41 Peptides**

Peptide on Plate	Antibody Bound (O.D. 450nm)		
	No Antibody	2F5 (10^{-9}M)	2F5 (10^{-8}M)
No Peptide	0.032	0.068	0.389
Control Peptide	0.019	0.055	0.425
Reduced Peptide	0.018	0.429	1.716
Native Peptide 1	0.024	0.558	1.301
Native Peptide 2	0.035	0.528	1.702

b. Reduced peptide bond based Aß transition state analogs: A reduced peptide bond linkage can be easily placed at any site in the Aß molecule (Fig. 7). The first reduced peptide bond transition state Aß analog that we made is the Gln-Lys-Leu-Val-Phe-**CH_2-NH_2^+**-Phe-Ala-Glu-Asp-Val-Gly-Cys-amide central region peptide; [calcd 1,342 (M+1), found 1,344]. Synthesis was begun using Fmoc chemistry, a commercially available Boc-Phe-**CH_2-NH_2^+**-Phe dipeptide was then added manually and automated synthesis was resumed to complete the peptide (60).

A structural comparison (Fig. 12) was made between the native Aß peptide and the reduced peptide bond transition state Aß analog using a graphics workstation. An energy minimization algorithm (2000 iterations) was applied to arrange each peptide in its most favorable conformation.

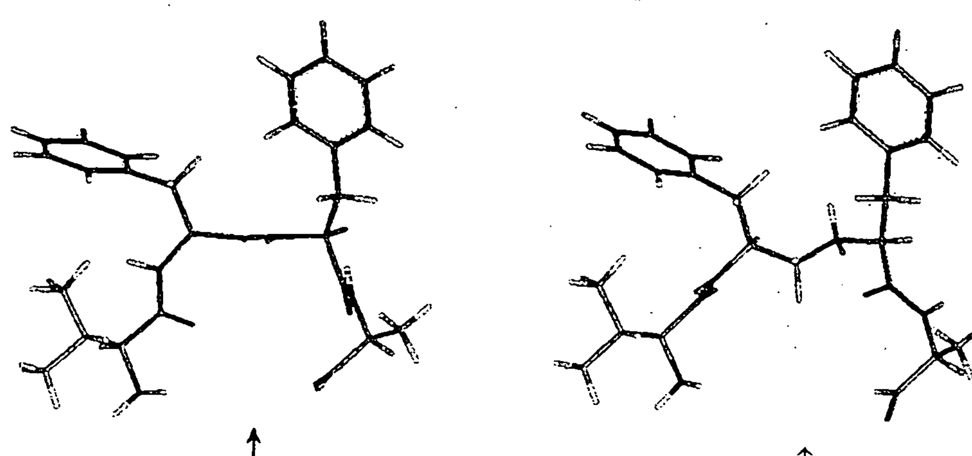

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Fig. 12 Val-Phe-CO-NH-Phe-Ala Val-Phe-CH_2-NH_2^+-Phe-Ala

The peptide link **-CO-NH-** (↑) between Phe_{19} and Phe_{20} of Aß was replaced with a reduced peptide bond **-CH_2-NH_2^+-** (↑) and an energy minimization was applied. This orientation shows the difference between the planar peptide link **-CO-NH-** (↑) of natural Aß (left) versus the corresponding tetrahedral moiety **-CH_2-NH_2^+-** (↑) in the reduced peptide bond transition state analog (right).

C.3 Produce Phosphonate or Phosphonamidate Transition State Analogs of gp41 and Aß

a. Phosphonamidate and phosphonate gp41 transition state analog peptides: The structures shown in Fig. 13 represent the putative transition state for peptide hydrolysis by zinc peptidases and the phosphonate or phosphonamidate mimic. Similar tetrahedral transition state intermediates are formed in each of the four classes of proteolytic enzymes, the serine-, cysteine-, aspartic- and metallo-peptidases.

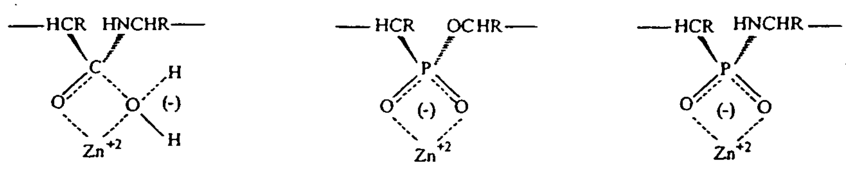


Fig. 13 Transition-state Phosphonate Phosphonamidate

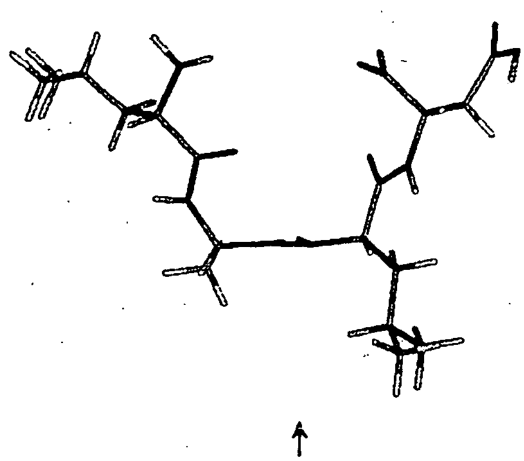
We have begun to synthesize a phosphonate transition state peptide analog of a naturally occurring close homolog Glu_{662}→Ala_{662} variant of the gp41 ELDKWA neutralization epitope, Ala-Leu-Asp-Lys-Trp-Ala (Fig. 8). Replacement of the proposed scissile peptide linkage between Ala_{662} and Leu_{663} (↓) with a phosphonate moiety (-***PO_2^--O***-) or phosphonamidate bond (***-PO_2^--NH-***) is designed to elicit catalytic antibodies that will hydrolytically cleave the virus at this site in the gp41 neutralization region of HIV.

Conformationally altered peptides will be created by incorporating either a phosphonate or phosphonamidate bond into the otherwise normal ALDKWA sequence of amino acids which comprise the critical gp41 neutralization determinant. Complementary antibody combining sites elicited by such peptides should recognize and selectively bind to that naturally-occurring epitope on the HIV envelope. However the native ELDKWA sequence in gp41 will necessarily be strained into a transition state-like conformation to attain congruency with those antibody sites. That steric alteration should make the antibody-bound epitope susceptible to hydrolytic cleavage, especially if the adjacent antibody side groups are favorable to acid-base catalysis.

Our scheme for the synthesis of the phosphonate analog is as follows. The N-terminal portion of the phosphonate peptide (Cys-Gln-Gln-Leu-Leu) was synthesized using standard automated Fmoc chemistry. Its amino terminus was capped by reaction with acetic anhydride while the peptide was still attached to the resin. After cleavage from the resin the N-acetyl pentapeptide was treated with pyridine disulfide to protect its sulfhydryl group.

The dimethyl phosphonate component (Ala-***PO_2^--O***-Leu) is being made using (-)-2-hydroxyisocaproic acid according to a previously reported seven-step synthesis with only slight modification (16). HBTU-activated peptide linkage (61) will be used to couple this amino-phosphonate and the N-acetyl pentapeptide. The HPLC-purified product will be treated with alkaline phosphatase plus rabbit liver esterase to remove the two methyl groups and yield N-acetyl-Cys-Gln-Glu-Leu-Leu-Ala-***PO_2^--O***-Leu. The free carboxyl on Leu affords an opportunity to extend the epitope sequence by coupling Asp-Lys-Trp-Ala-amide. Each component in the synthetic scheme will be tested for purity by HPLC and its composition will be verified by mass spectral and amino acid analysis. Treatment of the peptide with mercaptoethanol will generate a free sulfhydryl to facilitate thioether linkage of the transition state peptide to a maleimide activated KLH.

A structural comparison was made between the native peptide and the transition state peptide using a Silicon Graphics Iris Indigo XS24 graphics workstation (Fig. 14). An energy minimization algorithm (1000 iterations) was applied to the peptides to arrange each in its most favorable conformation.



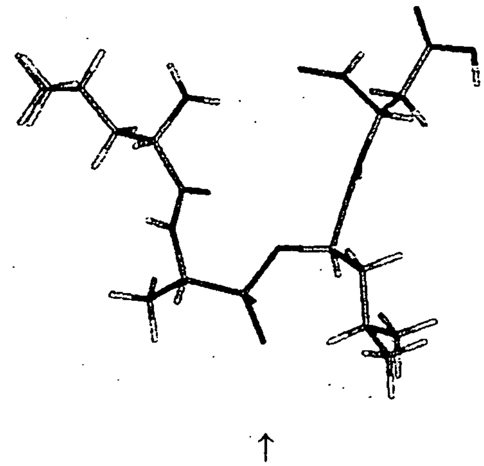


Fig. 14 Leu-Ala-CO-NH-Leu-Asp Leu-Ala-PO_2^--O-Leu-Asp

The peptide link ***-CO-NH-*** (↑) between Ala_{662} and Leu_{663} will be replaced with a phosphonate bond ***-PO_2^--O-*** (↑). The orientation shown above illustrates the difference between a planar peptide link of the native peptide (left) versus the corresponding tetrahedral phosphonate bond in the transition state peptide (right).

We have already synthesized a V3 transition state phosphonate peptide (N-acetyl-Cys-Gly-Pro-Gly-Arg-Ala-***PO_2^--O***-Phe) according to a scheme similar to that described above. Each component in the synthetic route was tested for purity by HPLC and its composition was verified by mass spectral and amino acid analysis.

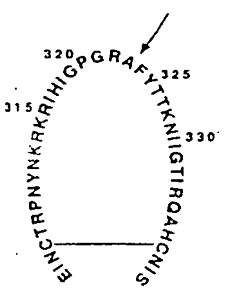


Fig. 14

Replacement of the proposed scissile peptide linkage between Ala_{323} and Phe_{324} (↓) with a phosphonate moiety (***-PO_2^--O-***) is designed to elicit catalytic antibodies that will hydrolytically cleave the virus at this site in the V3 loop (Fig. 15). The conformationally altered heptapeptide has been created by incorporating a phosphonate bond into the otherwise normal sequence of amino acids which comprise the critical crown region of the V3 loop. The complementary antibody combining sites elicited by such peptides should recognize and selectively bind to that naturally-occurring epitope on the HIV envelope. However the native sequence in V3 will necessarily be strained into a transition state-like conformation to attain congruency with those antibody sites. That steric alteration should make the antibody-bound epitope susceptible to hydrolytic cleavage, especially if the adjacent antibody amino acid side chain groups are favorable to acid-base catalysis.

b. Phosphonamidate and phosphonate Aß transition state analogs: A phosphonamidate transition state analog of the carboxy-terminal region of Aß has been synthesized (Fig. 16).

↓

N-acetyl-Cys-Met-Val-Gly-Gly--**CO-NH**--Val-Val-Ile-Ala-amide

35 40

Fig. 16 N-acetyl-Cys-Met-Val-Gly-Gly-**PO_2^--NH**-Val-Val-Ile-Ala-amide

Replacement of the proposed scissile peptide linkage between Gly_{38} and Val_{39} (↓) with a phosphonamidate moiety (-**PO_2^--NH**-) is designed to elicit catalytic antibodies that will hydrolytically cleave Aß at this site. The N-acetyl-Cys residue was placed at the position of Leu_{34} to provide a suitable linkage group for coupling this peptide to an antigenic carrier protein. The structures in Fig. 13 represent the putative transition state for peptide hydrolysis by zinc peptidases and the phosphonate and phosphonamidate mimics. Similar tetrahedral transition state intermediates are formed in each of the four classes of proteolytic enzymes.

The design strategy and methods for synthesizing phosphonamidate- and phosphonate-based transition state peptides are straightforward (62, 63). The N-terminal portion of the peptide (N-acetyl-Cys-Met-Val-Gly) was made using standard automated Fmoc chemistry. After cleavage from the resin the N-acetyl tetrapeptide was treated with pyridine disulfide to protect its sulfhydryl group. An acid chloride of Cbz-glycine phosphonate monomethyl ester (62, 63) was coupled with Val-Val-Ile-Ala-amide which was synthesized by automated Fmoc chemistry. The product, Cbz-Gly-PO_2^--NH-Val-Val-Ile-Ala-amide has a phosphonamidate (methyl ester) bond between the Gly and Val residues. Next, the Cbz blocking group was removed using hydrogen so that the protected N-acetyl-Cys-Met-Val-Gly peptide could be added to the amino terminal end of this transition state peptide by HBTU-activated peptide linkage. Treatment with mercaptoethanol and rabbit liver esterase was used to deblock the peptide. Each key component in the synthetic scheme was tested for purity by HPLC and its composition was verified by mass spectral and amino acid analysis. This new Aß analog, N-acetyl-Cys-Met-Val-Gly-Gly-**PO_2^--NH**-Val-Val-Ile-Ala-amide (Fig. 16) is designed to elicit catalytic antibodies that will specifically cleave Aß at the Gly-Val bond.

The synthesis of phosphonate Aß transition state analog peptide (eg. N-acetyl-Cys-Met-Val-Gly-Gly-**PO_2^--O**-Val-Val-Ile-Ala-amide) will follow a similar scheme and will use some of the same intermediates described for the phosphonamidate transition state analog.

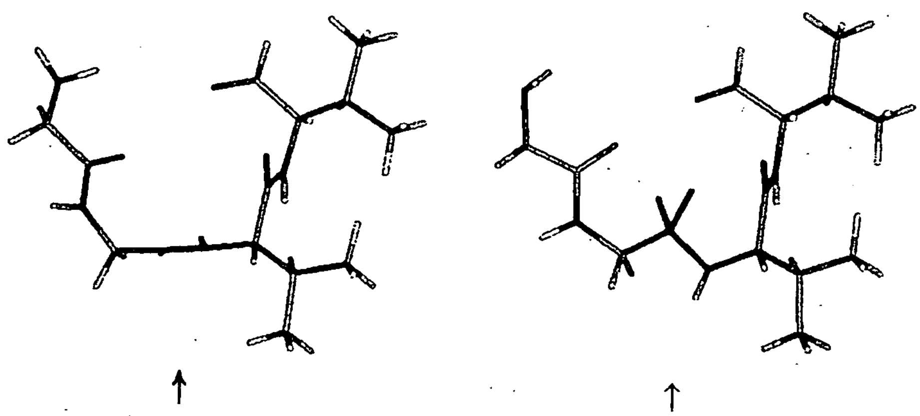


Fig. 17 Gly-Gly-CO-NH-Val-Val Gly-Gly-PO_2^--NH-Val-Val

A structural comparison was made between the native Aß peptide and the transition state phosphonamidate Aß peptide (Fig. 17) using a graphics workstation. The peptide link **-CO-NH-** (↑) between Gly_{38} and Val_{39} was replaced with a phosphonamidate bond **-PO_2^--NH-** (↑) and an energy minimization was applied. The orientation shown above illustrates the difference between the planar peptide link **-CO-NH-** (↑) of native Aß (left) versus the corresponding tetrahedral phosphonamidate bond **-PO_2^--NH-** (↑) in the transition state peptide (right).

An antibody combining site complementary to the tetrahedral transition state analog on the right of Fig. 16, will force the normally planar bond of the Aß substrate peptide on the left into a transition state-like conformation. Such bond distortion can catalyze the hydrolytic cleavage of the Aß peptide at the Gly_{38}-Val_{39} linkage.

C.4 Elicit Monoclonal Antibodies with the Novel Transition State Analog Peptides

a. Immunization of mice: The transition state analog gp41 and Aß peptides have been linked to an antigenic Keyhole Limpet Heamocyanin (KLH) carrier protein. A Cys residue was strategically placed at the N- or C-terminal end of those transition state peptides to provide a suitable linkage group for coupling them via a thioether bond to maleimide activated carrier proteins. This linkage is stable and attaches the peptide in a defined orientation.

Standard protocols were used to immunize BALB/c mice with the KLH-linked transition state analog peptides. Briefly this procedure used i.p. injection of the different antigens emulsified in

complete Freunds adjuvant, followed by a second course in incomplete adjuvant. Three days prior to the hybridoma fusion, the BALB/c mice were boosted i.v. with antigen in PBS.

For example, the Cys-Leu-Leu-Glu-***Sta***-Asp-Lys-Trp-Ala-Ser peptide (Fig. 3) which is a statine transition state analog of the ELDKWA gp41 determinant was linked to KLH. We emulsified this antigen in complete Freunds adjuvant and injected the preparation i.p. into mice. A very early bleed was examined for the presence of anti-transition state peptide antibodies that will recognize and potentially cleave the gp41 neutralization epitope. We screened the diluted sera from 4 immunized mice for reactivity with both the statine peptide (Sta-Peptide) and native peptide directly adsorbed onto ELISA wells (Table III).

Table III **ELISA for Binding to the Statine Versus Native gp41 Peptide**

	Antibody Bound (O.D. 450nm)	
Addition	**Sta-Peptide**	**Native Peptide**
Control Serum	0.059	0.112
Mouse 1 antiserum	2.460	0.350
Mouse 2 antiserum	2.772	0.365
Mouse 3 antiserum	2.388	0.323
Mouse 4 antiserum	2.479	0.417

The serum antibodies bound to these carrier-free ELDKWA gp41 peptides adsorbed directly to the microtitre plate, confirming their anti-peptide specificity. The analyzed sera preferentially recognized our statine gp41 transition state peptide but cross-reacted with the native gp41 peptide (Table III). In three weeks the animals will be boosted with antigen in incomplete Freunds adjuvant so that a more mature antibody population is simulated. This will give high affinity antibodies having greater cross-reaction with the native ELDKWA determinant. Spleen cells from transition state peptide-immunized mice with the highest titre sera will be fused with myeloma NS-1 cells to establish hybridomas according to standard procedures. The production of monoclonal antibodies has been a mainstay of this laboratory for many years and fusions are ordinarily run on a routine basis.

b. Hybridoma production: We performed a hybridoma fusion using the spleen of a mouse immunized with the phenylalanine statine transition state Aß-KLH antigen (Fig. 9). Monoclonal antibodies from some of hybridoma supernatants produced were screened using ELISA to assess their binding to both the normal $A\beta_{1-43}$ peptide and to the phenylalanine statine transition state Aß peptide. Two major patterns were found (Fig. 18).

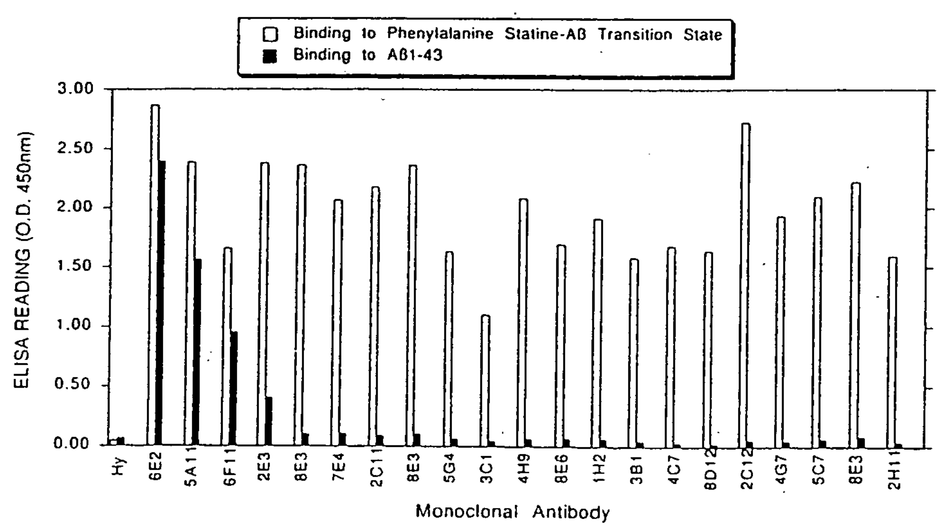


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Fig. 18 ELISA Comparing Antibody Binding to Transition State Versus Native Aß

One group of antibodies (at the left of Fig. 18) bound to the immunizing transition state peptide and cross-reacted strongly with the native $A\beta_{1-43}$ peptide when each was adsorbed directly

onto the ELISA plate. A second group (at the right) showed a high binding preference for the phenylalanine statine transition state Aß peptide and reacted minimally with native $Aß_{1-43}$.

Strong color reactions were obtained in this ELISA using only 10 µl of hybridoma supernatent while Hy media alone or PBS gave a low background (Fig. 18). These results demonstrate that the comparative ELISA screen, although only a semi-quantitative measure of binding, will provide a means for choosing monoclonal antibodies that are highly selective for, and most reactive with, the transition state. Importantly, the antibodies bound to the carrier-free Aß peptides adsorbed directly onto microtitre plates, showing their anti-peptide specificity.

These findings indicate that several of the new anti-Aß transition state antibodies are unique. They can bind to both the phenylalanine statine- and normal-Aß peptides. Their selective recognition of the transition state and weaker cross-reaction with native $Aß_{1-43}$ however implies that this binding interaction is very different from that shown by conventional anti-native Aß antibodies. It suggests further that these new antibodies may be able to force the native Aß peptide into a conformation resembling the transition state for hydrolytic cleavage.

A second distinct hybridoma fusion was performed using the spleen of a mouse immunized with a KLH conjugate of the statine (**Sta**) transition state analogs encompassing the carboxy-terminal region of Aß (Fig. 8). ELISA was used to demonstrate antibody binding to both the normal $Aß_{1-43}$ peptide and to the statine transition state Aß peptide (Fig. 19).

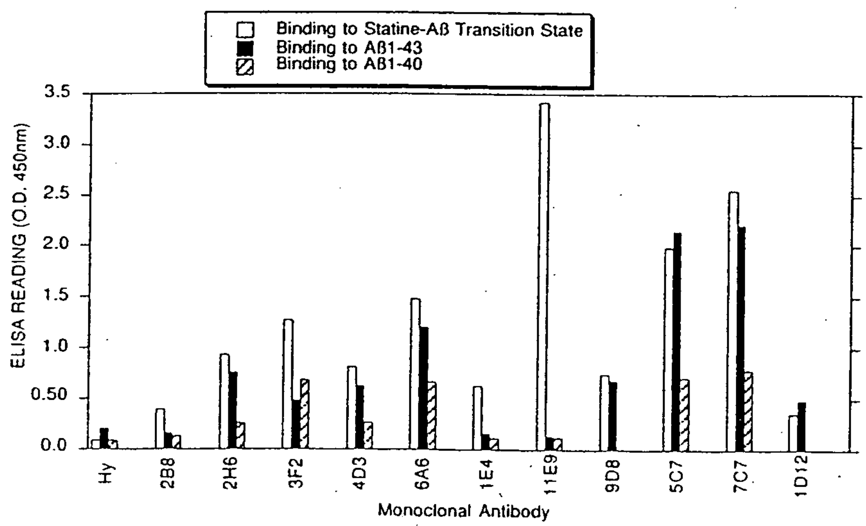


Fig. 19 ELISA Comparing Antibody Binding to Transition State Versus Native Aß

The antibodies bound to the C-terminal locus on these carrier-free Aß peptides adsorbed directly to the microtitre plate, confirming their anti-peptide specificity. Most of the antibodies preferentially recognized the statine Aß transition state but cross-reacted with native $Aß_{1-43}$. This suggests that these new antibodies may be able to force the native Aß peptide into a conformation resembling the transition state for hydrolytic cleavage of its C-terminal amino acids. Such cleavage would in effect convert $Aß_{1-43}$ into potentially less harmful shorter peptides, like $Aß_{1-40}$ or $Aß_{1-39}$.

Clone 11E9 had the strongest preference for the statine analog and might be the best prospect for having catalytic activity (Fig. 19). Several clones displayed no difference in their reactivity with the native versus statine transition state Aß peptide. We also tested the clones with $Aß_{1-40}$ to identify antibodies which do not react with this shortened, 40 amino acid version of Aß (Fig. 19). Used therapeutically, such antibodies would preferentially bind/cleave the less abundant but more noxious $Aß_{1-43}$ species in the blood as opposed to the smaller and less detrimental $Aß_{1-40}$.

A third hybridoma fusion was performed using a mouse immunized with the V3 phenylalanine statine transition state peptide-KLH antigen (Fig. 6). Monoclonal antibodies from the hybridoma supernatants were screened using ELISA to assess their binding to both the normal V3 loop peptide and the phenylalanine statine transition state V3 peptide. Data shown in Fig. 20 indicate that two different patterns of specificity are easily discernable. One group of antibodies bound almost equally well to both the immunizing transition state peptide and the native V3 peptide attached directly to the plate. Another group displayed a strong preference for the phenylalanine

Binding of Phenylalaninestatine Monoclonal Antibodies to V3 Peptides

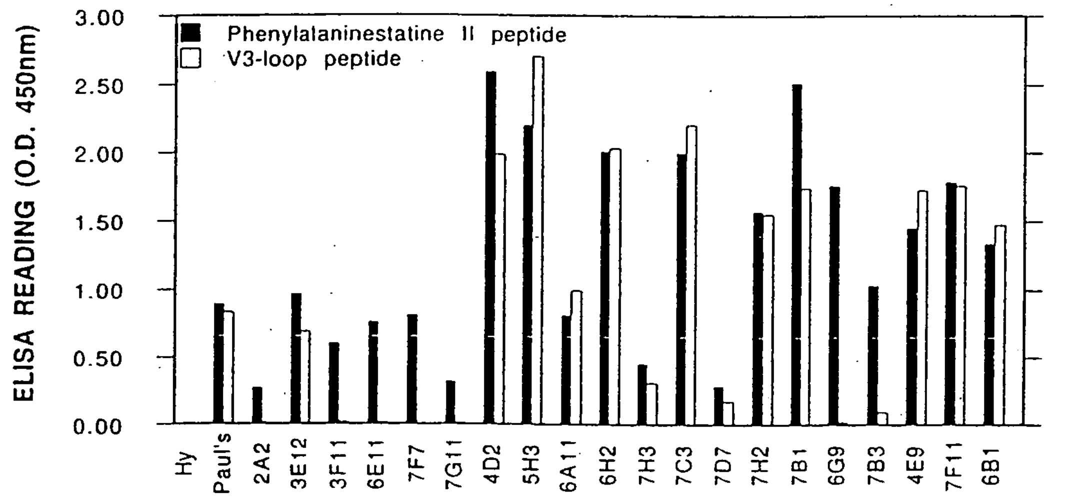
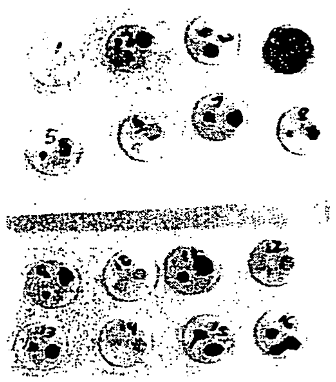


Fig. 20 ELISA Comparing Antibody Binding to Transition state Versus Native V3 Peptides

statine transition state peptide, e.g. 3F11, 6E11, 7F7, 7G11, 6G9 and 7B3 and these are the most likely candidates for having catalytic activity.



Importantly, the anti-transition state antibodies tested positive in an immunoblot assay that used two different preparations of pure, recombinant gp120 (glycosylated and non-glycosylated, from the AIDS Reference Reagent Program) spotted onto nitrocellulose (Fig. 21). Additional studies using flow cytofluorimetry showed that some of these antibodies reacted with 8E5 cells (64, 65) bearing budding HIV particles on their membrane (data not shown). This indicates that recognition of the native V3 epitope on the gp120 envelope protein is retained by the anti-transition state antibodies.

Blot 1, no antibody control;

Blot 2, anti-gp120 positive control;

Fig. 21 Blot on gp120 Blots 3-16, various anti-V3 transition state peptide clones.

An example of the types of antibodies that we expect to obtain from our phosphonate-based transition state immunogens can be had by examining the results from a forth hybridoma fusion performed using a mouse immunized with the V3 phosphonate peptide-KLH antigen (Fig. 14). Monoclonal antibodies from several of the hybridoma supernatants produced were screened using ELISA to assess their binding to both normal V3 peptide and the phosphonate transition state V3 peptide. Data shown in Fig. 22 indicate that two distinct specificity patterns are discernable. One group of antibodies bound equally well to both the immunizing transition state peptide and the native V3 peptide when each was attached to ovalbumin. The second group showed a consistent preference for the phosphonate transition state V3 peptide and reflected the specificity ratio found in the immune serum. Several clones, 2G5, 1C1, 1E3 and especially 4B3, displayed a pronounced binding preference for the transition state peptide compared to the other antibodies assayed (Fig. 22).

The sample run in duplicate, labeled Paul's Ab, is a monoclonal antibody raised against the native V3 peptide and is reactive primarily with the GPGRAF crown epitope (66). As expected this monoclonal antibody did not cross-react with the steriochemically altered transition state peptide GPGRA-$\mathbf{PO_2^-}$-**O**-F but showed very strong binding to normal V3. Collectively, these findings clearly indicate that the new anti-V3 transition state antibodies are unique. They can recognize both the phosphonate- and normal- V3 peptides. Their unique cross-reaction with native V3, however, implies that this binding interaction is very different from that shown by conventional anti-native V3 antibodies. It suggests that these new antibodies may be able to force the native V3 peptide into a conformation resembling the transition state for hydrolytic cleavage.

Binding of Monoclonal Antibodies to V3 Peptides

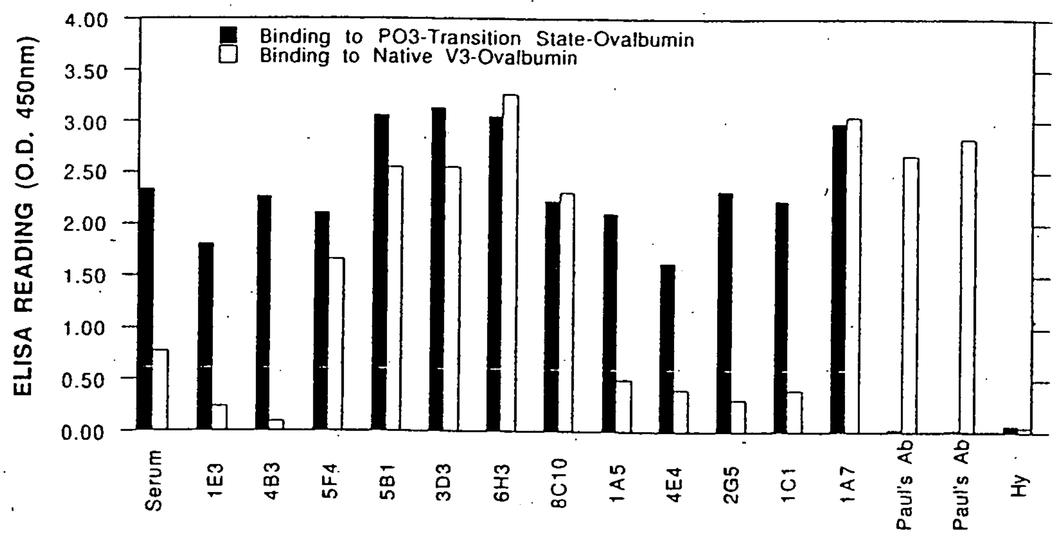


Fig. 22 ELISA Comparing Antibody Binding to Transition State Versus Native V3 Peptides

A predicted cleavage by-product peptide CGPGRA was synthesized and thioether-linked to maleimide activated ovalbumin so that it could be used for an ELISA that was strictly comparable to those presented in Fig. 22. Interestingly, monoclonal antibodies that recognized both the immunizing transition state peptide and the native V3 peptide equally well (Fig. 22) also cross-reacted with this shortened GPGRA cleavage peptide. In contrast, those antibodies which preferentially bound to the transition state peptide versus native V3 (clones 1E3, 4B3, 1A5, 4E4, 2G5 and 1C1) displayed no reactivity with the GPGRA in this ELISA. Thus we can now easily select clones so that product inhibition will not interfere with the antibody-mediated hydrolysis of the V3 loop.

These combined results demonstrate that the comparative ELISA screen will provide a means for choosing monoclonal antibodies that are highly selective for, and most reactive with our gp41 and Aß transition state peptides. Importantly, their anti-peptide specificity can be verified by reaction with the carrier-free peptides adsorbed directly onto microtitre plates. Those antibodies most likely to possess catalytic activity should preferentially bind the transition state peptide as opposed to the native peptide. Finally, any antibodies which strongly interact with expected cleavage products can be identified and discarded to avoid product inhibition of catalysis.

c. Screen antibodies using proteolytic assays: A solid phase ^{125}I-labeled Aß assay was developed to screen anti-transition state antibody hybridoma supernatants for specific proteolytic activity. The Cys-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Tyr-amide peptide encompassing amino acids 14-25 of Aß (Fig. 7) was synthesized with a Cys and Tyr added at either end. This was radiolabeled with ^{125}I and the iodinated peptide was then separated from unlabeled material by HPLC. The highly radioactive Aß peptide was coupled to a thiol-reactive, iodoacetyl-Sepharose gel to form an irreversible linkage. Catalytic antibodies should promote the progressive release of soluble ^{125}I-peptide from the solid phase matrix. The proposed assay was verified by the ability of several different proteases in to rapidly hydrolyze this Sepharose-linked Aß substrate. The peptide is readily accessible to proteolytic cleavage as revealed by a release of soluble ^{125}I-peptide that increased with incubation time (not shown). Selected antibodies were screened for catalytic activity using release of radioactivity from ^{125}I-Aß-Sepharose (Fig. 23).

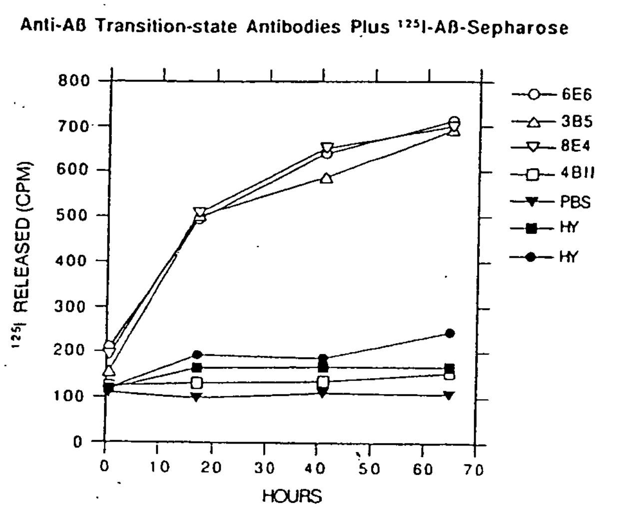


Fig. 23 Cleavage of ^{125}I-Aß-Sepharose

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The results obtained at pH 7, 25°C indicate that the antibody-containing media of several clones released ^{125}I-peptide at a greater rate than other clones from this fusion or the PBS and Hy medium controls (Fig. 23). Large amounts of these antibodies will now be obtained, purified and tested a higher concentrations to achieve much faster rates of cleavage and to verify that the antibodies are acting in a catalytic mode using conventional enzyme kinetics. By changing the composition of the ^{125}I-peptide we can use this same strategy to assay antibodies reactive with different regions of Aß.

We have also developed and used a similar solid phase assay to analyze anti-V3 transition state antibodies from one of our initial hybridoma fusions. An example of the kind of results that can be obtained is shown in Fig. 24. The preliminary data indicate that two anti-V3 clones, 7H3 and 3H3, released ^{125}I at a greater rate then the other antibodies or the controls, PBS and SHAM (eluate from protein A column after passage of medium with no antibody).

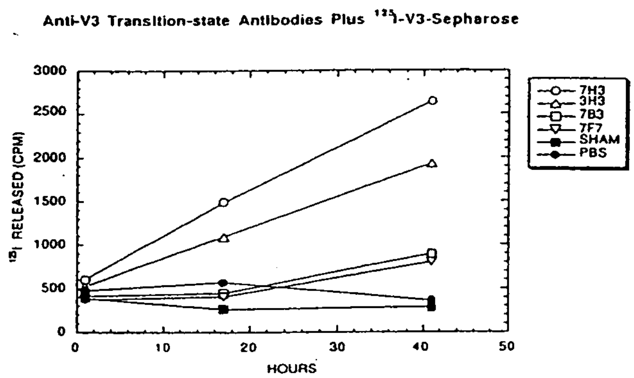

Fig. 24 Cleavage of ^{125}-I-V3-Sepharose

A problem with the solid phase screening assay is that it does not easily allow us to determine the identity of the cleaved ^{125}I-peptides or whether the release is truly due to antibody-mediated catalysis. We therefore devised a thin layer chromatography-based autoradiography assay so that we could obtain more definitive evidence for antibody-mediated cleavage of peptides.

The V3 system gives an example of how this assay operates. Selected anti-V3 transition state clones were expanded and used to induce ascites production in mice. The different monoclonal antibodies were then isolated using protein A-Sepharose. The cleavage assay used ^{125}I-V3 native peptides. One of these, N-acetyl-C-K-R-I-H-I-G-P-G-R-A-F-^{125}I-Y-T-T-K-amide, was the same length as the transition state V3 peptide used for immunization. We also made a smaller heptapeptide version of the crown epitope, N-acetyl-G-P-G-R-A-F-^{125}I-Y-amide. These two ^{125}I-V3 peptides bound to the appropriate anti-V3 transition state monoclonal antibodies when examined using either a PEG precipitation assay (Table IV) or by a co-electrophoresis method.

To test for peptide cleavage we added the antibodies to the ^{125}I-peptide, allowed them to incubate and then spotted the reaction mix onto polyamide thin layer sheets. The sheets were developed in different solvents (eg. 0.5N HCl, 0.5N NaOH or pH7 phosphate buffer) and the migration of ^{125}I-products was followed by exposing the sheet using a digitized phosphorimager system.

Table IV ^{125}I-V3 Peptide Binding By Anti-Transition State V3 Monoclonal Antibodies

Addition	^{125}I-V3 Heptamer Bound (cpm)	^{125}I-V3 16-mer Bound (cpm)
Control	6,429	19,317
Control	5,948	16,747
7B1	44,013	37,474
3H3	72,375	38,423
7B3	5,356	42,571

The action of naturally occurring proteases on the V3 peptides was also examined by this procedure. An example of the results of one of these experiments with the small N-acetyl-G-P-G-R-A-F-^{125}I-Y-amide peptide is shown in Fig. 25.

It is encouraging to see that these antibodies break down the V3 peptide compared to the untreated peptide (PBS in Fig. 25). The various enzymes were tested so that we could identify the cleavage site of the antibodies by comparison with the known specificity of the different enzymes. Obviously, more experiments must be done and additional controls will have to be run before we can conclusively state that the antibodies are catalytically hydrolyzing the V3 peptide at the right site.

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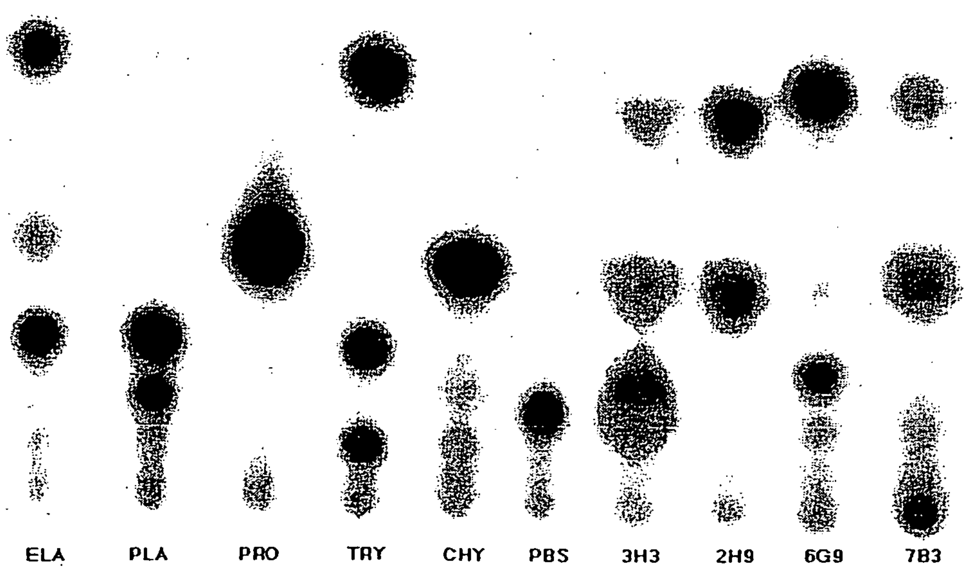

Fig. 25 TLC; ELA=elastase, PLA=plasmin, PRO=pronase, TRY=trypsin, CHY=chymotrypsin, PBS=control peptide alone, 3H3, 2H9, 6G9, 7B3 =different anti-V3 transition state antibodies.

An analogous thin layer chromatography-based autoradiography system will be used to assay the action of anti-ELDKWA catalytic antibodies on the radioactive C-L-L-E-L-D-K-W-A-^{125}I-Y peptide. We expect to get cleavage patterns comparable to those shown in Fig. 25, although the migration of those ^{125}I-peptide fragments should vary to some degree. The radioactive spots will be eluted and subjected to automated Edman degradation sequence analysis so that the exact locus of cleavage can be defined.

It is important to mention that the direct binding of native and transition state ^{125}I-peptides to antibodies can be measured by equilibrium dialysis or by polyethylene glycol precipitation methods (Table IV). This will provide a quantitative measure of affinity and will also allow for comparative inhibition studies that will augment the specificity data obtained by ELISA.

We also devised a thin layer chromatography-based autoradiography assay so that more definitive evidence for antibody-mediated cleavage of Aß could be obtained. We expanded selected anti-phenylalanine statine Aß transition state clones, induced ascites production and isolated the different monoclonal antibodies using protein A-Sepharose. The cleavage assay used ^{125}I-Aß$_{1-40}$ and a 17-mer, encompassing amino acids 9-25 (Fig. 7). These two ^{125}I-labeled peptides bound to the purified monoclonal antibodies 5A11 and 6E2 when examined using either a PEG precipitation assay or by a co-electrophoresis method. To test for peptide cleavage we added the antibodies to the ^{125}I-peptides, allowed them to incubate and then spotted the reaction mix onto polyamide thin layer sheets. The chromatographs were developed in different solvents (eg. 0.5N HCl, 0.5N NaOH or pH7 phosphate buffer) and the migration of ^{125}I-products was followed by exposing the sheet using a quantitative phosphorimager system (Fig. 26).

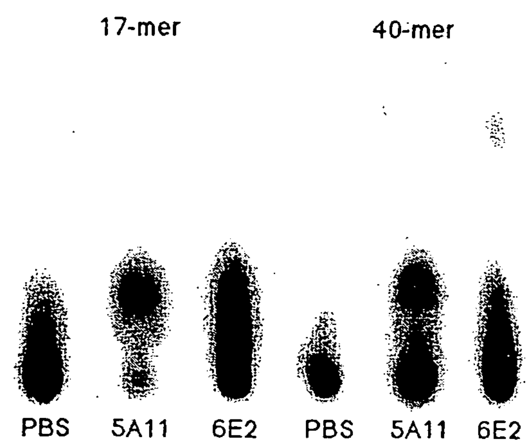

Fig. 26 TLC of ^{125}I-Aß-Cleavage

It is promising to see that these antibodies break down the Aß peptides compared to the untreated peptides (PBS). Clearly, more experiments must be performed and additional controls will have to be run before we can conclusively state that the antibodies are catalytically hydrolyzing the Aß peptide at the right site. Various naturally occurring proteases will be tested in this system so that we can identify the cleavage site of the antibodies by comparison with the known specificity of the different enzymes. We will also sequence the cleaved Aß peptides.

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D. RESEARCH DESIGN and METHODS

D.1 Synthesis of Statine Transition State Peptide Analogs of HIV gp41 and Aß

a. Statine based transition state analog gp41 peptides: The strategy and techniques for synthesizing statine based transition state peptides are provided in depth within the Preliminary Studies, Section C.1a. We have furnished details on the synthesis of a statine analog of the gp41 ELDKWA determinant. A mass spectrum of that new peptide was provided to verify its composition and give an indication of its purity (Fig. 4). Placement of a statine residue at alternative sites in gp41 or in other HIV envelope proteins can be easily accomplished using a analogous approach. The peptide synthesizer, mass spectrometer and amino acid analyzer are located at our BBRI facility so that any additional peptides can be easily synthesized if and when they are needed.

The stereochemical differences between the statine analog and the natural ELDKWA peptide were examined by computorial analysis (Fig. 5). These data will provide an insight into the various conformations that might be recognized by monoclonal antibodies elicited with this transition state peptide as well as the degree of distortion or constraint that will be induced into the antibody-bound natural ELDKWA peptide. The free energy required to force the native peptide into a geometry resembling the transition state will be determined. Calculations will provide an estimate of the influence that this strain may have in terms of its association binding constant (K_a) to an anti-transition state antibody.

b. Statine based transition state analog Aß peptides: We synthesized both statine based and phenylalanine statine based transition state Aß peptides by standard automated Fmoc chemistry (Preliminary Studies, Section C.1b, Figs. 8 and 9). Stereochemical differences between the phenyalanine statine analog and natural Aß peptide were inspected by computorial analysis (Fig. 10).

D.2 Synthesis of Reduced Peptide Bond Transition State Analogs of HIV gp41 and Aß

a. Reduced peptide bond based transition state analog gp41 peptides: Synthesis of a reduced peptide bond based transition state analog of the gp41 ELDKWA epitope has been accomplished (Preliminary Studies, Section C.2a). The stereochemical distinctions between that analog and the natural ELDKWA peptide were examined by computorial analysis (Fig. 11). Placement of a reduced peptide bond at alternative sites in gp41 or in other HIV envelope proteins can be easily accomplished using a analogous approach.

b. Reduced peptide bond based transition state analog Aß peptides: Synthesis of a reduced peptide bond based transition state analog of Aß has been accomplished (Preliminary Studies, Section C.2b). The stereochemical distinctions between that analog and the natural ELDKWA peptide were examined by computorial analysis (Fig. 12). Placement of a reduced peptide bond at alternative sites in Aß can be easily accomplished using similar approachs.

D.3 Synthesis of Phosphonate or Phosphonamidate Analogs of HIV gp41 and Aß

a. Phosphonate or phosphonamidate analog gp41 peptides: The basic design strategy and methods for synthesizing phosphonate based ELDKWA transition state peptides are provided in detail under the Preliminary Studies, section C.3a and Fig. 14. Placement of a phosphonate linkage at alternative sites in gp41 or in other HIV proteins could be accomplished in a similar manner if this was deemed necessary or advantageous.

The chemical methods for synthesizing phosphonamidate based transition state peptides are straightforward (62, 63). We will couple an acid chloride of Cbz-leucine phosphonate monomethyl ester with Asp-Lys-Trp-Ala-Ser-amide to produce (Cbz-Leu-**PO_2^--NH**-Asp-Lys-Trp-Ala-Ser-amide) with

a phosphonamidate bond (**-PO_2^--NH-**) between the highly conserved Leu and Asp. The Cbz blocking groups will be removed so that N-acetyl-Cys-Leu-Leu-Glu can be added to the amino terminal end of the transition-state peptide. This new ELDKWA analog, N-acetyl-Cys-Leu-Leu-Glu-Leu-**PO_2^--NH**-Asp-Lys-Trp-Ala-Ser-amide is designed to elicit catalytic antibodies that will recognize and cleave the conserved gp41 ELDKWA epitope of HIV. Placement of a phosphonamidate linkage at alternative sites in gp41 or in other HIV proteins could be accomplished in a similar manner. Unlike phosphonate derivatives, these phosphonamidate compounds can be prone to hydrolysis. Therefore, the stability at neutral pH of these new phosphonamidate peptide analogs will be evaluated by HPLC analysis.

b. Phosphonate or phosphonamidate analog Aß peptides: We have synthesized a phosphoamidate based transition state Aß peptide by combined organic synthesis and standard automated Fmoc chemistry (Preliminary Studies, Section C.3b, Fig. 16). The stereochemical differences between the phosphoamidate analog and the natural Aß peptide were examined by computorial analysis (Fig. 17). Placement of phosphonate or phosphoamidate linkages at alternative sites in Aß would be carried out using the different protocols described above or in Section C.3.

D. 4 Production of Monoclonal Antibodies Using the Novel Transition State Analog Peptides

a. Antigen synthesis and immunization protocols: The transition state analog gp41 and Aß peptides will be linked to antigenic carrier proteins like KLH or ovalbumin in order to elicit an immune response. A Cys residue will be strategically placed at the N- or C-terminal end of the peptides to provide a suitable linkage group for coupling them via a thioether bond to maleimide activated carrier proteins. This stable linkage attaches the peptide in a defined orientation. Addition of ~20 peptides/KLH has been obtained based upon the transition state amino acid content as determined by amino acid analysis of the hydrolyzed conjugates.

The transition state analog antigens will be emulsified in complete Freunds adjuvant and injected i.p. into BALB/c mice. After ~1 month animals will be given a boost i.p. using the antigen emulsified with incomplete Freunds adjuvant. Serum from these animals will be analyzed for anti-peptide antibodies by ELISA (Table III).

b. Hybridoma fusion and ELISA screening: BALB/c mice showing abundant antibody production will be boosted by an i.v. injection with antigen and three days later they will be used to generate hybridoma clones that secrete monoclonal antibodies. Spleen cells from mice with the highest titre will be fused with mouse myeloma NS-1 cells to establish hybridomas according to standard procedures (68, 69). Monoclonal antibody production has been a mainstay of our laboratory for many years and fusions are routinely run on a regular basis. We have made substantial progress toward obtaining several different types of anti-transition state analog monoclonal antibodies (Preliminary Studies Section, C.4).

The initial screen for anti-transition state peptide analog monoclonal antibodies will be performed using ELISA (Preliminary Studies Section, C.4b). Both the transition state peptide and the corresponding natural ELDKWA or Aß peptide will be adsorbed onto separate microtitre plates. The hybridoma supernatants will be screened using two assays so that the relative binding to both native and transition state peptides can be quantitated. Clones producing monoclonal antibodies that preferentially recognize the transition state analog will be selected for expansion and further study.

When searching for potential catalytic antibodies, the clones will be secondarily screened by ELISA to detect and discard antibodies with substantial affinity for the predicted cleavage products of the native peptide. This is important since such catalytic antibodies would be subject to product inhibition and might therefore exhibit low turnover. For example, antibody-mediated cleavage of the HIV gp41 ELDKWA or ALDKWA native peptides would yield LLEL and DKWA for the anti-statine antibodies (Fig. 3) or QLLA and LDKWA for the anti-phosphonate transition state antibodies (Fig. 14). Correspondingly, the native Aß peptides would yield MVGG and VVIAT for the anti-phosphonate or anti-phosphoamidate antibodies (Fig. 16); HQKLVF and FAEDVG for the anti-phenylalanine statine transition state antibodies (Fig. 9); GGVV and IAT for the anti-statine antibodies (Fig. 8).

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c. Screening hybridoma supernatants for catalytic activity: As numerous monoclonal antibodies are generated against our gp41 ELDKWA and Aß transition state peptides we will need to screen the most promising ones for catalytic activity. A rapid intitial test for catalytic antibodies will be performed using high specific activity solid phase assays (Preliminary Studies, Section C.4c and Figs. 23, 24). Terminal cysteine and tyrosine containing peptides will be synthesized and then radiolabeled with ^{125}I. The iodinated peptide will be separated from unlabeled material by HPLC to achieve essentially quantitative specific activity (~2000 Ci/mmol). For example we have recently synthesized a C-L-L-E-L-D-K-W-A-^{125}I-Y peptide which will be used to screen our anti-gp41 specific statine, reduced peptide bond and phosphonate/phosphoamidate clones for catalytic activity.

The radioactive peptides will be coupled to a thiol-reactive, iodoacetyl-Sepharose gel to form an irreversible linkage. Release of radioactivity from this matrix will be used to assay anti-transition state antibodies for catalytic activity. Tests can be performed directly on antibodies in the hybridoma culture medium or antibody can be isolated using protein A-Sepharose and assayed at pH7, 25oC with the solid phase peptide. These Sepharose-linked, ^{125}I-peptide substrates will provide a sensitive and convenient method for detecting catalytic cleavage by the progressive release of soluble ^{125}I-peptide. Preliminary assays indicate that interference from high levels of background hydrolysis is not a problem when testing hybridoma supernatants of clones that do not produce catalytic antibodies. As a further precaution against exogenous proteases, all hybridoma cell fusions and cell culturing can be carried out in serum-free media.

Analysis of the catalytic activity of the anti-transition state antibodies will also be obtained using a thin layer chromatography-based autoradiography system (Preliminary Studies, Section C.4c and Figs. 25, 26). A soluble radioactive peptide will be used as the substrate and the separation of cleavage fragments is achieved on polyamide sheets. Data are collected and quantified using a digitized phosphorimager system. The locus of cleavage will be defined by comparison with known enzyme cleavage sites and by analysis of the radioactive peptide spots using an automated sequencer.

A fluorogenic assay will also be used to detect antibody-dependent peptidase activity. Commercially available amino acid 7-amido-4-methylcoumarin derivatives will be used as a soluble, fluorogenic substrates to test for cleavage on the carboxyl-side of the scissile bond. Hydrolytically released 7-amino-4-methylcoumarin is highly fluorescent (1nM is detectable) so these substrates or modifications thereof provide a simple, but sensitive, direct fluorometric assay.

d. Isolation of selected monoclonal antibodies: While we expect that the highly sensitive assays developed (see section **c**) will allow us to screen for catalytic antibody activity at the hybridoma stage, a high concentration of purified monoclonal antibody might be required to measure ELDKWA epitope or Aß cleavage. Several criteria will be used to determine which hybridoma clones to expand and use for ascites production in pristane primed mice. These would include, a strong binding preference for the transition state analog versus native peptide, no binding to the cleavage product peptides and reactivity with whole virus or full-length Aß. Ascites will be collected and the antibodies will be purified by $(NH_4)_2SO_4$ precipitation and passage over an S-300 column to isolate the 150 kDa immunoglobulin fraction. Monovalent Fab fragments will be prepared and isolated by established methods. Protein A Memsep and DEAE Memsep columns will be used on a ConSep liquid chromatography system to prepare highly purified monoclonal antibodies and Fab fragments which must be free of extraneous protease activity. Their purity will be evaluated by SDS-PAGE under reducing and non-reducing conditions. Any residual proteases will be removed by passage over immobilized proteinase inhibitors.

e. Characterization of the antibodies and the cleavage products: With purified anti-transition state antibodies in hand, we will proceed to characterize their binding specificity, the mechanism of antibody-dependent peptide cleavage as well as the resulting peptide and viral cleavage products. Direct binding of the ^{125}I-peptides to the antibodies will be measured at 4° C by equilibrium dialysis or by polyethylene glycol precipitation methods (Table IV). These assays are extremely sensitive and, by using serial dilution, will provide relative binding affinities for the different hybridoma supernatants or purified antibodies. Competitive displacement assays will be used to measure the relative binding strength of different unlabeled peptides. This will provide a quantitative measure of affinity to augment the specificity data obtained by ELISA.

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A detailed analysis of the catalytic activity of the anti-transition state antibodies will be obtained using a thin layer chromatography-based autoradiography system (Preliminary Studies,

Section C.4c and Figs. 25, 26). Radioactive peptides will be used as the substrate the cleavage site will be defined by comparison with known enzyme cleavage sites and by analysis of the radioactive peptide spots by automated Edman sequence determination.

Several important variables will be examined for their impact on the catalytic activity of the anti-transition state antibodies, including pH, temperature, ionic strength and the presence of different metal ions. Thus for example, specific cleavage will be assayed at three pH values, pH 5, pH 7 and pH 9 either at 25° or 37° C. Zn^{++}, Cu^{++} and other metals that can facilitate peptide hydrolysis will be tested for effects on the antibody catalyzed reaction.

General assays for detecting the proteolytic cleavage of any of the synthetic peptides would include HPLC analysis or TLC followed by detection of the newly generated peptides using the highly sensitive fluorescamine reagent. Cleavage of ^{125}I-labeled peptides will be discerned by autoradiography following separation by polyamide TLC using several solvents.

Many of the assays and conditions detailed in the Preliminary Studies Section for measuring catalytic activity on gp41 and Aß peptide substrates will be employed to fully define the hydrolytic properties of the isolated anti-transition state antibodies. As described, a comparison will be made with the uncatalyzed reaction run under identical conditions. At this stage, however, some very important controls will be run. First we will ensure that catalytic antibody activity is completely blocked by the appropriate transition state peptide. This non-cleavable "inhibitor" should bind much more tightly to the antibody combining sites and thereby prevent substrate binding or cleavage. Substrate specificity will be further established by showing no cleavage of a sham gp41 and Aß peptides having a different amino acid sequence. The products of hydrolysis will be fully characterized by HPLC, amino acid and mass spectral analysis. Control antibodies that are not directed against the transition state analog ELDKWA or Aß peptides will be tested and are expected to produce no catalysis. Catalytic activity will be shown to reside in the purified Fab fragments of the anti-transition state antibodies.

A kinetic analysis of the catalyzed reaction will be performed by measuring the initial rates of hydrolysis as a function of substrate concentration. Data will be analyzed on a Lineweaver-Burke plot so that it can be confirmed that the reaction follows classical Michaelis-Menten kinetics. A rate acceleration for hydrolysis will be calculated by the ratio of k_{cat}/k_{uncat}. The reaction will also be run for an extended length of time to demonstrate catalytic turnover.

In addition to demonstrating binding to and cleavage of soluble Aß peptides, we will also explore the effect of the purified catalytic anti-Aß antibodies on insoluble Aß precipitates (23, 24). These Aß aggregates form spontaneously *in vitro* and closely mimic the amyloid plaques found in the brain of Alzheimer's patients. A highly active catalytic antibody might destroy insoluble Aß plaques by hydrolytically cleaving the constituent aggregated peptides. Moreover, if insoluble peptide is in equilibrium with a low level of soluble Aß, then a catalytic anti-Aß antibody could cleave the soluble component, upset this balance and gradually dissolve the precipitate. These interesting possibilities will be tested since Aß precipitates can be easily formed and measured *in vitro* (8, 9).

Identification of catalytic anti-Aß antibodies with the capacity to dissolve Aß aggregates will be performed using a radioactive solubilization assay. We have set up this radioactive test as a quick way to screen the different monoclonal antibodies produced in our laboratory for an ability to dissolve preformed Aß aggregates. After adding ^{125}I-Aß to unlabeled soluble peptide, aggregates were formed by bringing the solution to pH 5 or by stirring it overnight in PBS. An aliquot of this labeled aggregate will be incubated for 1hr with either PBS, the catalytic anti-Aß antibody or an equal amount of an irrelevant mouse antibody. After centrifugation, the level of radioactivity in the precipitate versus the supernatant will be measured to calculate the percent of Aß solubilized.

Since the catalytic anti-gp41 antibodies are projected for eventual clinical usage, it will be important to demonstrate their reactivity with trimeric gp41 domains and whole HIV particles. Two gp41 ectodomain peptides, N-51 and C-43 will be synthesized using automated Fmoc chemistry. These peptides will be mixed to form a stable, alpha-helical trimer of heterodimers which is believed to be the core of the fusion-competent state of the HIV envelope (39). We will validate trimer formation by analytical ultracentifugation at our in-house physical biochemistry facility at BBRI. The ELDKW epitope is located at the carboxyl terminus of the C-43 part of this complex. Antibody binding to this 34 kDa trimeric complex versus the 6 kDa C-43 monomer will be tested by ELISA, immunoblot assay

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and by co-migration on gel electrophoresis. Reduced temperatures may be required to limit catalytic cleavage of the epitope. It will be interesting to determine if antibody binding/cleavage affects complex stability or formation.

Recognition of oligomeric forms of gp41 by our antibodies will also be assayed by immunoblot analysis of PAGE-separated viral complexes prepared by boiling viral lysates in the presence of low SDS concentrations (69). Binding of anti-ELDKWA transition state monoclonal antibodies to whole HIV particles will also be assayed by ELISA. This system uses sucrose density isolated virus, ($\sim 10^{11}$ particles/ml) from Advanced Biotechnology, captured onto plates using an anti-gp120 antibody (43).

A cytofluorographic (FACS) assay will be used to test for the specific attachment of anti-ELDKWA transition state antibodies to budding HIV particles on the surface of 8E5 cells which constitutively express a non-infective form of HIV. There is a complete in-house FACS facility at BBRI and we have many years experience using this methodology.

In addition to testing binding to and cleavage of the ELDKWA peptide, we will evaluate if the antibodies display comparable activity with the 34 kDa trimeric complex of N-51 and C-43 which may be the core of the fusion-competent state of the HIV envelope (39). C-43 will be labeled with ^{125}I at its single tyrosine residue and a complex with N-51 will then be formed. This radiolabeled complex will be incubated with the catalytic antibody for different time intervals, the reaction will be stopped and then the protein will be separated by size using SDS PAGE. The gel will then be analyzed by autoradiography using a phosphoimaging system. Catalytic antibody-treated reaction mixtures should yield a faster migrating band in different proportions dependent on incubation time.

The catalytic antibodies will also be tested for effects on whole HIV-1 particles both free and on the surface of cells. Sucrose density isolated virus ($\sim 10^{11}$ particles/ml) can be obtained from Advanced Biotechnologies. Human 8E5 cells produce high level of non-infectious HIV particles that bud into the medium from their membrane (64, 65) and will therefore provide a good source of both free and cell-bound virus for evaluating the action of catalytic antibodies. Size differences resulting from cleavage will be monitored by immunoblotting SDS PAGE separated fragments using appropriate peroxidase-labeled, HIV-specific antibodies as a detection system. We have obtained 8E5 cells, gp41 peptides, monoclonal anti-gp41 antibodies and many other HIV-related reagents from the AIDS Reference Reagent Program.

Syncytia formation and p24 production assays (64, 65, 70-72) can be used as a measure of the biological functioning of HIV. Thus, we can test our hypothesis that catalytic antibodies, by permanently cleaving the ELDKWA epitope, should be far superior to conventional anti-ELDKWA antibodies for neutralizing HIV infectivity. Exponentially growing CEM-SS cells will be bound to poly-L-lysine-coated microtitre wells. The cells will be exposed to ~100 syncytium-forming units of untreated virus, anti-ELDKWA antibody-treated virus or catalytic anti-ELDKWA antibody-treated virus for 1h. Following removal of the supernatent, fresh medium will be added and then the plates will be incubated for 4 days at 37°C. Viral p24 protein will be quantified (Coulter Source, Inc) on day 4 using 50µl of supernatant medium. Syncytia will be enumerated microscopically on day 5. Neutralization titres for the various antibodies will be determined by the point of 50% inhibition of p24 production or syncytium formation compared to untreated virus. Standard laboratory strains of HIV will be used for these assays but exceptional inhibition by a catalytic antibody will be confirmed using primary isolates and normal cells as targets (73). We can also obtain virology support to help evaluate the effects of our antibodies on HIV inactivation by collaborating with other research groups in the field. For example, the appendix contains a letter of support from our collaborators (74-78) in the Virology Research Group at Sydney.

Time Table for "Novel Transition State Peptide Analog Antigens"

The major elements of **Specific Aim A1 (Produce Statine Transition State Peptide Analogs of HIV gp41 and Aß)** are well underway. Synthesis of a unique statine-based ELDKWA transition state analog peptide has already been completed. A series of novel central region Aß phenylalanine statine analogs were also synthesized. In addition we have made a series of carboxyl-terminal Aß statine peptides. Some of the details regarding the characterization of these new statine transition state constructs should be finished in Year 1. As the need arises, additional statine transition state peptide analogs with modifications placed at alternative target sites in the ELDKWA epitope or in the Aß sequence can be synthesized during Years 2-4.

We have made substantial progress on the major elements of **Specific Aim A2 (Produce Reduced Peptide Bond Transition State Analogs of HIV gp41 and Aß)**. Recently we completed the synthesis of reduced peptide bond analogs of both the gp41 ELDKWA epitope and Aß. Two different approaches were taken and analogous chemistry will allow placement of this novel modification at any position in a peptide sequence. As the need arises, additional reduced peptide bond transition state peptide analogs with modifications placed at alternative target sites in the ELDKWA epitope or in the Aß sequence can be synthesized during Years 2-4.

Work on the major elements of **Specific Aim A3 (Produce Phosphonate or Phosphonamidate Analogs of HIV gp41 and Aß)** is proceeding smoothly. We have gained substantial experience synthesizing both phosphonate and phosphonamidate peptide analogs. A phosphonamidate Aß analog has already been made in our lab. The ongoing production of an ALDKWA peptide having a tetrahedral phosphonate bond between Ala_{662} and Leu_{663} is high priority and should also be completed in Year 1. As the need arises, additional phosphonate or phosphonamidate transition state peptide analogs with modifications placed at alternative target sites in the ELDKWA epitope or in the Aß sequence can be synthesized during Years 2-4.

We have begun to generate of some of the monoclonal reagents outlined in **Specific Aim A4 (Elicit Monoclonal Antibodies with the Novel Transition State Analog Peptides)**. Specific Aim 4 gets to the heart of the matter and therefore every effort will be made to move these experiments forward as quickly as possible. Several different gp41 and Aß transition state analog peptides have been coupled to a KLH carrier protein to create antigen vaccines designed to elicit catalytic antibodies. These novel antigens have been injected into mice and each has produced a good antibody response. A comparative ELISA screening protocol has been developed to detect the most promising anti-transition state analog monoclonal antibodies. Sensitive proteolytic assays have been devised to screen hybridoma supernatants for catalytic activity. To optimize our chances for obtaining the best possible reagents we will examine antibodies elicited by each of the different ELDKWA and Aß transition state analogs. Multiple hybridoma fusions will be performed using different mice immunized with each antigen that we have produced. We already have gained considerable experience in producing and analyzing anti-transition state monoclonal antibodies. That background should help to expedite this phase of our research. The majority of this monoclonal antibody work will be carried out over the course of Years 1-4.

During this phase we will also fully characterize the catalytic antibodies, their interaction with naturally occurring substrates and the composition of the resulting cleavage products. Functional assays will also be utilized at this stage. Anti-Aß antibodies will be tested for their ability to dissolve plaque-like Aß aggregates formed *in vitro*. Antibody-mediated cleavage of whole HIV particles will be examined. Outstanding anti-gp41 catalytic antibodies also will be provided to our collaborators so that they can be tested for their ability to inhibit viral infection using syncytia formation and p24 assays. Most of these studies are expected to be performed in Year 5. This final year is also requested to allow us to recognize and solve any unexpected problems which may arise.

E. HUMAN SUBJECTS None

F. VERTEBRATE ANIMALS

The BBRI-ERI Joint Animal Facilities conform to Federal Guidelines (Guide for the Care and Use of Laboratory Animals) and has NIH approval (Sept. 1991-Jan.1996). The Animal Care and Use Committee reviews all protocols annually.

Balb/c mice, 6-10 weeks old are the animals which will be used for this project. Approximately 100 Balb/c mice will be used in the first 12-month period. The Balb/c mice are required for the generation of catalytic anti-gp41 and catalytic anti-Aß monoclonal antibodies and for ascites production, which are an ongoing needs for this research. The restraining holders used during injections are comfortable for the animals and not overly restrictive.

Inhalation of carbon dioxide is the method of euthanasia which will be used. This method is consistent with recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

Veterinary and diagnostic services are provided by the Tufts University School of Veterinary Medicine in Jamaica Plain, Massachusetts through contract with the Angell Memorial Hospital, Robert Hopkins, DVM.

The principal investigator, laboratory personnel, and animal technicians involved in the proposed research have had instruction or have demonstrated their competence in the care, use and handling of laboratory animals. We give assurance of humane practice in animal maintenance and experimentation; and we subscribe to the concept of using every acceptable method in the performance of this project to minimize the use of animals and to prevent animal distress.

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H. CONSULTANTS None

I. CONTRACTUAL ARRANGEMENTS None

CHECKLIST

TYPE OF APPLICATION *(Check all that apply.)*

[X] NEW application. *(This application is being submitted to the PHS for the first time.)*

[ ] REVISION of application number:
(This application replaces a prior unfunded version of a new, competing continuation, or supplemental application.)

[ ] COMPETING CONTINUATION of grant number:
(This application is to extend a funded grant beyond its current project period.)

INVENTIONS AND PATENTS *(Competing continuation appl.only)*
[ ] No
[ ] Yes. If "Yes," [ ] Previously reported [ ] Not previously reported

[ ] SUPPLEMENT to grant number:
(This application is for additional funds to supplement a currently funded grant.)

[ ] CHANGE of principal investigator/program director.
Name of former principal investigator/program director:

[ ] FOREIGN application or significant foreign component.

1. ASSURANCES/CERTIFICATIONS

The following assurances/certifications are made and verified by the signature of the Official Signing for Applicant Organization on the Face Page of the application. Descriptions of individual assurances/certifications begin on page 27 of Section III. If unable to certify compliance where applicable, provide an explanation and place it after this page. •Human Subjects; •Vertebrate Animals; •Debarment and Suspension; •Drug-Free Workplace (*applicable to new [Type 1] or revised [Type 1] applications only)*; •Lobbying; •Delinquent Federal Debt; •Research Misconduct; •Civil Rights (Form HHS 441 or HHS 690); •Handicapped Individuals (Form HHS 641 or HHS 690); •Sex Discrimination (Form HHS 639-A or HHS 690); •Age Discrimination (Form HHS 680 or HHS 690); •Financial Conflict of Interest.

2. PROGRAM INCOME *(See instructions, page 20.)*

All applications must indicate whether program income is anticipated during the period(s) for which grant support is requested. If program income is anticipated, use the format below to reflect the amount and source(s).

Budget Period	Anticipated Amount	Source(s)

3. INDIRECT COSTS

Indicate the applicant organization's most recent indirect cost rate established with the appropriate DHHS Regional Office, or, in the case of forprofit organizations, the rate established with the appropriate PHS Agency Cost Advisory Office. If the applicant organization is in the process of initially developing or renegotiating a rate, or has established a rate with another Federal agency, it should, immediately upon notification that an award will be made, develop a tentative indirect cost rate proposal. This is to be based on its most recently completed fiscal year in accordance with the principles set forth in the pertinent *DHHS Guide for Establishing Indirect Cost Rates,* and submitted to the appropriate DHHS Regional Office or PHS Agency Cost Advisory Office. Indirect costs will *not* be paid on foreign grants, construction grants, grants to Federal organizations, grants to individuals, and conference grants. Follow any additional instructions provided for Research Career Awards, Institutional National Research Service Awards, and specialized grant applications.

[X] DHHS Agreement dated: January 27, 1998 [ ] No Indirect Costs Requested

[ ] DHHS Agreement being negotiated with ______ Regional Office

[ ] No DHHS Agreement, but rates established with ______ Date ______

CALCULATION* *(The entire grant application, including the Checklist, will be reproduced and provided to peer reviewers as confidential information. Supplying the following information on indirect costs is optional for forprofit organizations.)*

a. Initial budget period: Amount of base $ 137,851 x Rate applied 92 % = Indirect Costs (1) $ 126,823

b. Entire proposed project period: Amount of base $ 746,646 x Rate applied 92 % = Indirect Costs (2) $ 686,914

(1) Add to total direct costs from form page 4 and enter new total on Face Page, Item 7b.
(2) Add to total direct costs from form page 5 and enter new total on Face Page, Item 8b.

*Check appropriate box(es):

[X] Salary and wages base [ ] Modified total direct cost base [ ] Other base (Explain)

[ ] Off-site, other special rate, or more than one rate involved (*Explain*)

B000569

Explanation *(Attach separate sheet, if necessary.)*:

4. SMOKE-FREE WORKPLACE

Does your organization currently provide a smoke-free workplace and/or promote the nonuse of tobacco products or have plans to do so?

[X] Yes [ ] No *(The response to this question has no impact on the review or funding of this application.)*

/

Remember to N-acetylate this peptide !!

Boston Biomedical Research Institute
Peptide Synthesis Requisition Form
ABI 431A Synthesizer

Please complete the following form and submit it to Anna Wong for each peptide to be synthesized.

17 mer

Date 3/16/98

Investigator Name: Vic Raso Ext# 316

Department:

Sequence (use 3 letter code):

H_2N- Cys - Tyr - Glu - Val - His - His - Gln - Lys - Leu - Val -
- Phe - Phe - Ala - Glu - Asp - Val - Gly
-COOH

10 - 25

The Amino Acids in stock are:

Fmoc-L-Ala	Fmoc-Gln(Trt)	Fmoc-L-Leu	Fmoc-L-Ser(tBu)
Fmoc-L-Arg(Pmc)	Fmoc-L-Glu(OtBu)	Fmoc-L-Lys(Boc)	Fmoc-L-Thr(tBu)
Fmoc-Asn(Trt)	Fmoc-Gly	Fmoc-L-Met	Fmoc-L-Trp
Fmoc-L-Asp(OtBu)	Fmoc-L-His(Trt)	Fmoc-L-Phe	Fmoc-L-Tyr(tBu)
Fmoc-L-Cys(Trt)	Fmoc-L-Ile	Fmoc-L-Pro	Fmoc-L-Val)

Choose a Resin: Rink Resin (Amide) ✓ HMP Resin (Acid) ____

Choose a Scale: 0.10mmole ____ (for 20 residue, assuming 80-90% cleavage recovery, yields about 150-175 mg crude peptide)

0.25mmole ✓ (for 20 residue, assuming 80-90% cleavage recovery, yields about 390-440 mg crude peptide)

Do you want the N-Terminal Fmoc removed? Yes ✓ No ____

Additional Instructions

Operators Comments Flow Test OK, Started 4/3/98

Fees: Set-Up Charge $25.00
0.25mmole Scale $12.00/Amino Acid
0.10mmole Scale $10.00/Amino Acid

peptide was split - 1/2 acetylated 1/2 not

Cleavage of the peptide from the resin is not included in this service!!!

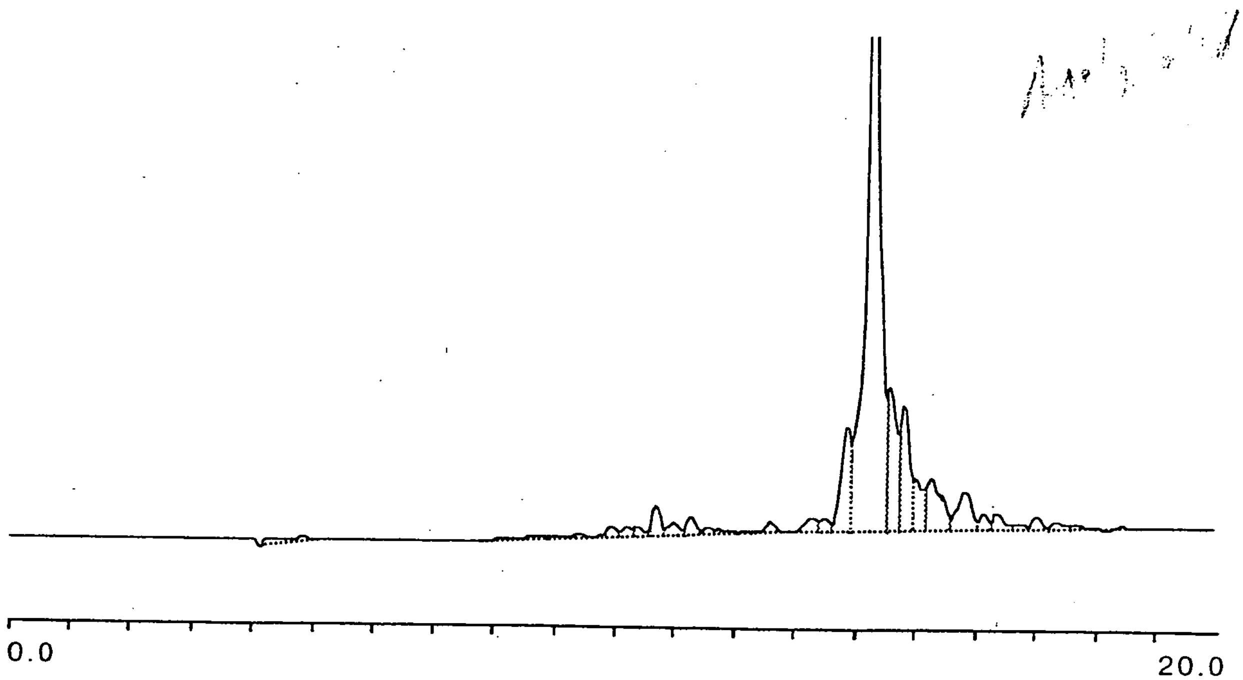
B000582

Date: Thu, Apr 9, 1998 9:25 AM
Data: pepanal 0-100-09APR98-005

Sample: Raso 17-mer acetylated 25μg injected
Column: C5 analytical 1ml/min
Buffer A: 0.1% TFA; Buffer B: 0.1%TFA in 99.9% CH_3CN
Gradient: 0-100%B, 20'
Monitor: 220nm, 2.0 AUFS; 280nm, 1.0 AUFS

Processing File: profile#1
Method: pepanal 0-100
Sampling Int: 0.1 Seconds

Data:



Analysis: Channel A

Peak No.	Time	Type	Height(μV)	Area(μV-sec)	Area%
1	4.833	N	5991	301853	1.373
2	8.685	N1	5299	108289	0.492
3	9.921	N4	12235	133552	0.607
4	10.216	N5	12034	121486	0.552
5	10.360	N6	13067	118242	0.538
6	10.668	N7	40475	296837	1.351
7	10.978	N8	15160	194175	0.883
8	11.258	N9	24391	203513	0.926
9	11.550	N10	6751	150316	0.684
10	12.593	N12	13337	101685	0.462

Date: Thu, Apr 9, 1998 9:25 AM
Data: pepanal 0-100-09APR98-005

Analysis: Channel A

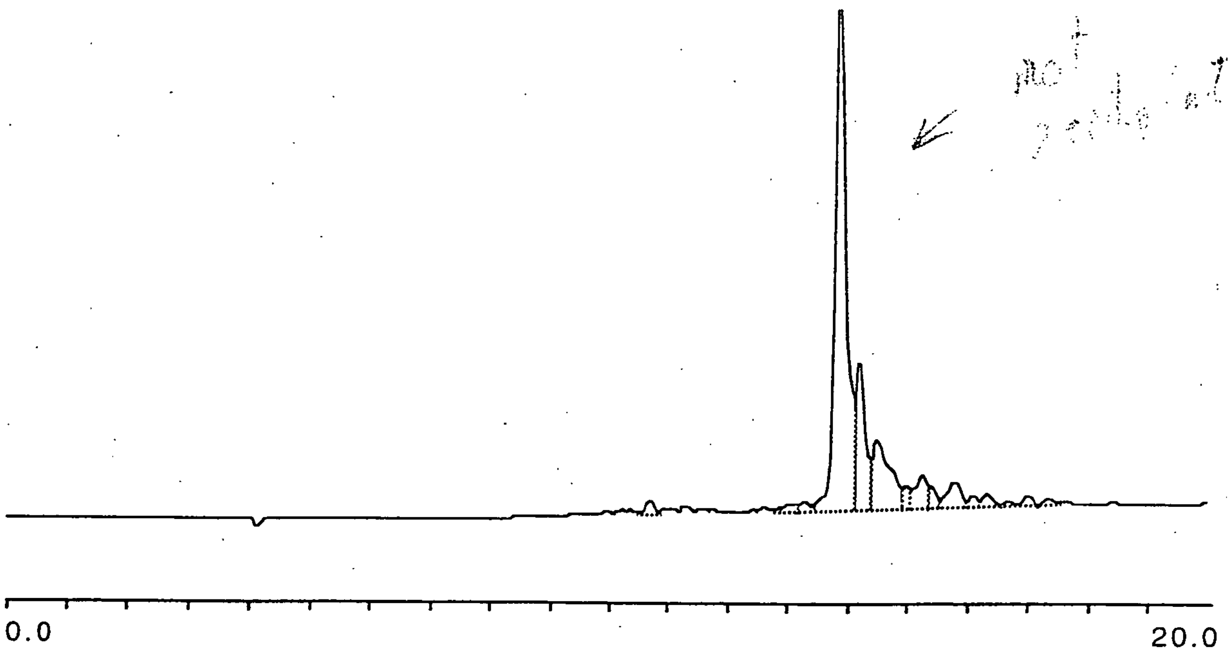
Peak No.	Time	Type	Height(μV)	Area(μV-sec)	Area%
11	13.285	N1	18399	203576	0.926
12	13.483	N2	15886	189637	0.863
13	13.853	N3	135926	1357611	6.179
14	14.181	N4	816316	10999738	50.069
15	14.533	N5	188584	2187892	9.959
16	14.786	N6	161526	1543324	7.025
17	14.975	N7	67265	817543	3.721
18	15.263	N8	67365	1150360	5.236
19	15.835	N9	50784	751768	3.421
20	16.120	N10	20463	197003	0.896
21	16.326	N11	22661	273588	1.245
22	16.696	N12	8780	111649	0.508
23	17.016	N13	18891	183284	0.834
24	17.353	N14	9565	135223	0.615
25	18.431	N15	4161	136780	0.622
Total Area				21968924	99.987

Date: Thu, Apr 9, 1998 8:49 AM
Data: pepanal 0-100-09APR98-004

Sample: Raso 17-mer unacetylated 25µg injected
Column: C5 analytical 1ml/min
Buffer A: 0.1% TFA; Buffer B: 0.1%TFA in 99.9% CH_3CN
Gradient: 0-100%B, 20'
Monitor: 220nm, 2.0 AUFS; 280nm, 1.0 AUFS

Processing File: profile#1
Method: pepanal 0-100
Sampling Int: 0.1 Seconds

Data:



Analysis: Channel A

Peak No.	Time	Type	Height(µV)	Area(µV-sec)	Area%
1	10.673	N7	16653	133269	0.975
2	13.088	N3	10678	163364	1.195
3	13.285	N4	11455	133139	0.974
4	13.815	N5	660099	7324481	53.586
5	14.166	N6	188926	1914334	14.005
6	14.466	N7	89294	1888231	13.814
7	14.970	N8	28986	236050	1.726
8	15.241	N9	42523	544355	3.982
9	15.381	N10	29351	253598	1.855
10	15.773	N11	33162	506888	3.708

Date: Thu, Apr 9, 1998 8:49 AM
Data: pepanal 0-100-09APR98-004

Analysis: Channel A

Peak No.	Time	Type	Height(µV)	Area(µV-sec)	Area%
11	16.100	N12	14828	139119	1.017
12	16.306	N13	16033	182751	1.337
13	16.996	N15	13680	131388	0.961
14	17.335	N16	8976	117539	0.859
Total Area				13668506	99.994

used to make ALZ-FF

Boston Biomedical Research Institute
Peptide Synthesis Requisition Form
ABI 431A Synthesizer

Please complete the following form and submit it to Anna Wong for each peptide to be synthesized.

Date 12/17/98

Investigator Name: Vic Raso Ext 316

Department:

Sequence (use 3 letter code):

added on to reduced peptide bond

H_2N- Gln - lys - Leu -

-COOH

The Amino Acids in stock are:

Fmoc-L-Ala	Fmoc-Gln(Trt)	Fmoc-L-Leu	Fmoc-L-Ser(tBu)
Fmoc-L-Arg(Pmc)	Fmoc-L-Glu(OtBu)	Fmoc-L-Lys(Boc)	Fmoc-L-Thr(tBu)
Fmoc-Asn(Trt)	Fmoc-Gly	Fmoc-L-Met	Fmoc-L-Trp
Fmoc-L-Asp(OtBu)	Fmoc-L-His(Trt)	Fmoc-L-Phe	Fmoc-L-Tyr(tBu)
Fmoc-L-Cys(Trt)	Fmoc-L-Ile	Fmoc-L-Pro	Fmoc-L-Val)

V-F-R-F-A-E-D-V-G-C

Choose a Resin: Rink Resin (Amide) ____ HMP Resin (Acid) ____

Choose a Scale: 0.10mmole ____ (for 20 residue, assuming 80-90% cleavage recovery, yields about 150-175 mg crude peptide)

0.25mmole X (for 20 residue, assuming 80-90% cleavage recovery, yields about 390-440 mg crude peptide)

Do you want the N-Terminal Fmoc removed? Yes X No ____

Additional Instructions use same protocol as amide resin MEOH wash.

i.e. deblock with pyridine first then add Leu

Operators Comments Resin added to vessel → swell; deprot.; couple; N-Term FMOC removed; MEOH dried.
12.17.98 Synth Begun.

Fees: Set-Up Charge $25.00
0.25mmole Scale $12.00/Amino Acid
0.10mmole Scale $10.00/Amino Acid

Alz FF
MW = 1340.7

Cleavage of the peptide from the resin is not included in this service!!!

B000605

/

used to make ALZ - FF

Boston Biomedical Research Institute
Peptide Synthesis Requisition Form
ABI 431A Synthesizer

Please complete the following form and submit it to Anna Wong for each peptide to be synthesized.

Date 12-8-98
Investigator Name: Vic Raso Ext# 316
Department:

Sequence (use 3 letter code):
H_2N-Ala-Glu-Asp-Val-Gly-Cys-amide

-COOH

added reduced peptide

The Amino Acids in stock are:

Fmoc-L-Ala	Fmoc-Gln(Trt)	Fmoc-L-Leu	Fmoc-L-Ser(tBu)
Fmoc-L-Arg(Pmc)	Fmoc-L-Glu(OtBu)	Fmoc-L-Lys(Boc)	Fmoc-L-Thr(tBu)
Fmoc-Asn(Trt)	Fmoc-Gly	Fmoc-L-Met	Fmoc-L-Trp
Fmoc-L-Asp(OtBu)	Fmoc-L-His(Trt)	Fmoc-L-Phe	Fmoc-L-Tyr(tBu)
Fmoc-L-Cys(Trt)	Fmoc-L-Ile	Fmoc-L-Pro	Fmoc-L-Val)

Choose a Resin: Rink Resin (Amide) X HMP Resin (Acid) ____

Choose a Scale: 0.10mmole ____ (for 20 residue, assuming 80-90% cleavage recovery, yields about 150-175 mg crude peptide)

0.25mmole X (for 20 residue, assuming 80-90% cleavage recovery, yields about 390-440 mg crude peptide)

Do you want the N-Terminal Fmoc removed? Yes X No ____

Additional Instructions deprotect → NMP washes → no DCM
→ NO MEOH

Operators Comments flow tests ok → 0.48 g MBHA Rink resin used.
end cycle NMP wash only → synthesis began 12.9.98

Fees: Set-Up Charge $25.00
0.25mmole Scale $12.00/Amino Acid
0.10mmole Scale $10.00/Amino Acid

AEDVGC

Cleavage of the peptide from the resin is not included in this service!!!

FDF

B000606

SynthAssist™ Peptide Synthesis

Report type: Sequence
12/9/98 @ 12:34 PM

File name:

Sequence editor:

Sequence : Ala-Glu-Asp-Val-Gly-Cys-[NH2]

Comments : Vic Raso
MBHA Rink resin 0.25mmole
N Terminal deprotect
No DCM/MeOH washes

Disulfide Bonds: 0
Chemistry : Fmoc

Composition:

Sequence : [H]-Ala-Glu-Asp-Val-Gly-Cys-[NH2]

Composition : C22 H37 N7 O10 S1
Weight : 591.6421
C-Terminal : NH2
N-Terminal : H
# of Residues : 6
AA List :

AA	Count
Ala	1
Asp	1
Cys	1
Glu	1
Gly	1
Val	1

Calculations:

(Calculation not updated)

/ —

Boston Biomedical Research Institute
Peptide Synthesis Requisition Form
ABI 431A Synthesizer

Please complete the following form and submit to Gina Pagani for each peptide to be synthesized.

Date: 7/23/99

Investigator: Vic Vinar(?) Ext #: 316

Department: ______ room #: ______

Peptide Name: ALZ 1-42

Sequence (please use one letter code and print clearly):

H$_2$N-D-A-E-F-R-H-D-S-G-Y-E-V-H-H-Q-K-L-V-F-F-A-E-D-V-G-S-N-K-G-A-I-I-G-L-M-V-G-G-V-V-I-A-COOH

The amino acids kept in stock are:

Ala.......A	Cys.......C	His.......H	Met.......M	Thr.......T
Arg.......R	Gln.......Q	Ile.......I	Phe.......F	Trp.......W
Asn.......N	Glu.......E	Leu.......L	Pro.......P	Tyr.......Y
Asp.......D	Gly.......G	Lys.......K	Ser.......S	Val.......V

Choose a resin: ☐ Rink Amide Resin ☒ HMP Acid Resin

Choose a scale: ☒ 0.25 mmole - for 20 residues, yields about 390-440 mg crude peptide, assuming 80-90% cleavage recovery.

☐ 0.10 mmole - for 20 residues, yields about 150-175 mg crude peptide, assuming 80-90% cleavage recovery.

disregard

Removal of N-Terminal Fmoc Group: Yes ☒ No ☐

Additional Instructions: I have had trouble with the 43-mer but not the 40 mer — maybe the resin should be split in half after amino acid #20.

Operator's Comments: 0.31 g mBHA rink flow tests ok
Synthesis begun 8.5.99 → remove 1/2 resin at cycle 21. ~~increase coupling of 41,42 (DC. Arg?)~~
4.5.00 → 0.18 g HMP 0.71 g Benz Ant. flow tests OK Synthesis begun 4.5.00
ext couple #35-#42 (D-D) BADEIIIIF I = 900 s.

Fees for non-BBRI research projects:

Set-up Charge	$25.00
0.25 mmole Scale	$12.00/amino acid
0.10 mmole Scale	$10.00/amino acid

✂ Cleavage of peptide from resin not included.

Boston Biomedical Research Institute
Peptide Synthesis Requisition Form
ABI 431A Synthesizer

Please complete the following form and submit to Gina Pagani for each peptide to be synthesized.

Date: 3/27/99

Investigator: Vic Rao   Ext #: 316

Department: ____   room #: ____

Peptide Name: ALZ 1-40

Sequence (please use one letter code and print clearly):

H_2N- N-A-E-F-R-H-D-S-G-Y-E-V-H-H-Q-K-L-V-F-F-A
E-D-V-G-S-N-K-G-A-I-I-G-L-M-V ~~-COOH~~ G-G-V-V

The amino acids kept in stock are:

Ala........A	Cys.......C	His.......H	Met.......M	Thr.......T
Arg.......R	Gln.......Q	Ile........I	Phe.......F	Trp.......W
Asn.......N	Glu.......E	Leu.......L	Pro.......P	Tyr.......Y
Asp.......D	Gly.......G	Lys.......K	Ser.......S	Val.......V

Choose a resin: ☐ Rink Amide Resin ☒ HMP Acid Resin

Choose a scale: ☒ 0.25 mmole - for 20 residues, yields about 390-440 mg crude peptide, assuming 80-90% cleavage recovery.

☐ 0.10 mmole - for 20 residues, yields about 150-175 mg crude peptide, assuming 80-90% cleavage recovery.

Removal of N-Terminal Fmoc Group: Yes ☒ No ☐

Additional Instructions: dry but don't cleave

Operator's Comments: 0.18 g HMP resin. 0.61 g Benzoic Anhydride to cap. Flowtests OK. synthesis began 4.27.99

Fees for non-BBRI research projects:

Set-up Charge	\$25.00
0.25 mmole Scale	\$12.00/amino acid
0.10 mmole Scale	\$10.00/amino acid

✂ Cleavage of peptide from resin not included.

B000649

Boston Biomedical Research Institute
Peptide Synthesis Requisition Form
ABI 431A Synthesizer

Please complete the following form and submit it to Anna Wong for each peptide to be synthesized.

10 mg

Date 3/31/98
Investigator Name: Vic Raso Ext#
Department:

Sequence (use 3 letter code):

H_2N- Tyr - Met - Val - Gly - Gly - Val - Val - Ile -
Ala - Thr
-COOH

The Amino Acids in stock are:

Fmoc-L-Ala	Fmoc-Gln(Trt)	Fmoc-L-Leu	Fmoc-L-Ser(tBu)
Fmoc-L-Arg(Pmc)	Fmoc-L-Glu(OtBu)	Fmoc-L-Lys(Boc)	Fmoc-L-Thr(tBu)
Fmoc-Asn(Trt)	Fmoc-Gly	Fmoc-L-Met	Fmoc-L-Trp
Fmoc-L-Asp(OtBu)	Fmoc-L-His(Trt)	Fmoc-L-Phe	Fmoc-L-Tyr(tBu)
Fmoc-L-Cys(Trt)	Fmoc-L-Ile	Fmoc-L-Pro	Fmoc-L-Val)

Choose a Resin: Rink Resin (Amide) ____ HMP Resin (Acid) X

Choose a Scale: 0.10mmole ____ (for 20 residue, assuming 80-90% cleavage recovery, yields about 150-175 mg crude peptide)

0.25mmole X (for 20 residue, assuming 80-90% cleavage recovery, yields about 390-440 mg crude peptide)

Do you want the N-Terminal Fmoc removed? Yes X No ____

Additional Instructions

Operators Comments Flow test OK, Started 4/9/98

Fees: Set-Up Charge $25.00
0.25mmole Scale $12.00/Amino Acid
0.10mmole Scale $10.00/Amino Acid

Cleavage of the peptide from the resin is not included in this service!!!

B000674

ABI MODEL 431A VERSION 2.00 Fmoc

SYNTHESIS REPORT

SEQUENCE NAME: V. Raso 10mer 0.25mmole

RESIN: HMP Resin = 0.227gm

OPERATOR: A. Wong

RUN EDITOR:

Cy: 1 Rpt: 1 M: hefffghefffg

Cy: 2 Rpt: 9 M: BADEFG

Cy: 11 Rpt: 1 M: bdc

ADD TIME HOLD CYCLE: 0

RESIN SAMPLES: No

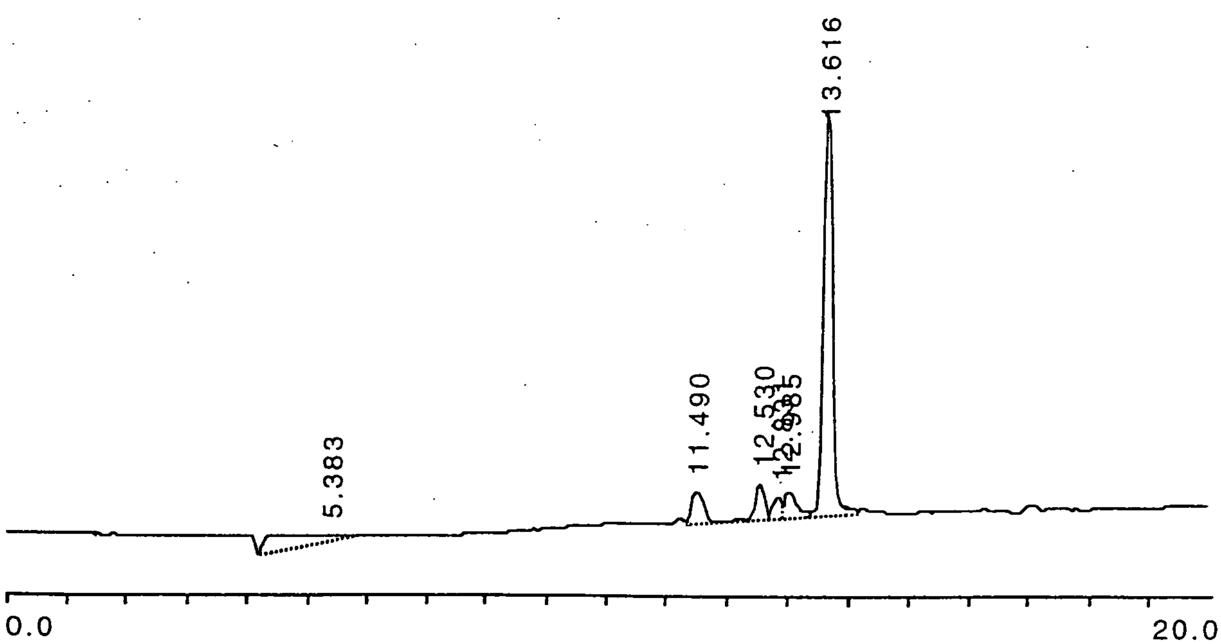
EVENTS LOG:		DATE	TIME	CYCLE	MOD	STEP	SEC REM
Synthesis begun		04/09/98	10:49	1	1	1	10
Barcode read	Thr	04/09/98	10:49	1	1	2	9
Barcode read	Spl	04/09/98	12:29	1	7	2	9
Barcode read	Ala	04/09/98	14:14	2	2	1	9
Barcode read	Ile	04/09/98	15:08	3	2	1	9
Barcode read	Val	04/09/98	16:02	4	2	1	9
Barcode read	Val	04/09/98	16:56	5	2	1	9
Barcode read	Gly	04/09/98	17:50	6	2	1	9
Barcode read	Gly	04/09/98	18:44	7	2	1	9
Barcode read	Val	04/09/98	19:39	8	2	1	9
Barcode read	Met	04/09/98	20:33	9	2	1	9
Barcode read	Tyr	04/09/98	21:27	10	2	1	9

Date: Tue, Apr 14, 1998 3:02 PM
Data: pepanal 0-100-14APR98-004

Sample: V. Raso 10mer peptide 25µg injected
Column: C8 analytical 1ml/min
Buffer A: 0.1% TFA; Buffer B: 0.1%TFA in 99.9% CH3CN
Gradient: 0-100%B, 20'
Monitor: 220nm, 2.0 AUFS; 280nm, 1.0 AUFS

Processing File: profile#2
Method: pepanal 0-100
Sampling Int: 0.1 Seconds

Data:



Analysis: Channel A

Peak No.	Time	Type	Height(µV)	Area(µV-sec)	Area%
1	5.383	N	3484	545738	15.148
2	11.490	N2	20948	217149	6.027
3	12.530	N5	22777	168299	4.671
4	12.831	N6	14168	114393	3.175
5	12.985	N7	17124	231470	6.425
6	13.616	N8	270268	2325480	64.551
Total Area				3602529	99.997

Alum 8/11/99

2% = 2 gms / 100 ml

20 mg / ml

~~1~~ 1 mg / 50λ binds ~ 100 ug Ag

use ~ 100 ug / mouse

make ~~a~~ a solution of AB_{1-40} @ 1 mg / ml

~~Take 50λ~~

2%

used 100λ of Alum + 100λ of AB_{40} = 100 ug

2 mg

for 3 mice

100λ

400λ of Alum + 400λ of AB_{40} = 400λ / 800λ

rotate for 30'

inject ~ 200λ / mouse

100 ug / 200λ

injected 3 mice 8/11/99

boost same 9/7/99

boost same 10/30/99

in PBS → boost I.P. 1/26/00

No Alum

boost I.P. 3/22/00

mouse died ~ 4 days later

500 mg / 3 ml = inject 0.6 ml

~ 200 mg/ml

Tg Mice

AA- 49/50

9/12/98 (49)/50 got CFA
(9/13/9)
10/24/98 IFA

7/27/99 IFA

/18/99 IFA

/24/00 IFA

120 + 120 PBS + 240 IFA =

use ~ 0.2/m

100 + 100 + 200 IFA

$\underbrace{30 + 30 + 30}_{90}$

+ 90 PBS + 180 IFA

1

AA 15

1/5/99

ALZ- reduced peptide

QKLVF-R-FAEDVGC amide -KLH

replaces serine 26

dialyzed vs PBS final 1mg/ml in nunc vial

do 2 mice

50 ug/mouse use 100ug

= 100ul + 200ul PBS + 300ul CFA

2/4/99 100ul + 200ul PBS + 300ul IFA

3/29/99 boost mice with 50ug in PBS i.p.

5/26/99 do 3 mice

150ul + 200ul PBS + 350ul CFA

6/19/99 boost with IFA

2/15/00 25 ug/mouse 1mg/ml 100ug

use 50ul + 250ul PBS + 300ul CFA

3/16/00 50ul + 250ul PBS 300ul of IFA

12/27/98

F®F. bought this from Bachem

ALZ- reduced F-®-F

Gln-Lys-Leu-Val-Phe-®-Phe-Ala-Glu-Asp-

Val-Gly-Cys-amide

MW = 1,341 + 1 = 1,342

got 1,344

use 13 mg KLH solids = 1mg = 1×10^{-9} moles KLH =

2.35×10^{-7} moles Mal groups

use ~ 0.315 mg = 1:1

↓

1mg

5/16/00

used 15 mg KLH solids = ~1mg = 1×10^{-9} moles KLH

2×10^{7} moles Mal

used ~ 0.5mg FF = 3×10^{-7} moles

= ~ 1:1

let go overnite

then dialyze

vs PBS

B000761

Alz Ntb D1

11/25/97 3D9 (D) 2 vials
3D9 (C) 2 vials

3/6/98 4D8 2 vials
9E7 2 vials

Alz 1-40

C 1
2/27/98

4E12	2 vials	7B12	2 vials
3C7	2 vials	9B11	2 vials
3G11	2 vials	7E6	2 vials
3H1	2 vials	10B6	2 vials
4E7	2 vials	10A4	2 vials
5G12	2 vials	5A9	2 vials
6A10	2 vials	10B3	2 vials
9C2	2 vials	7G1	2 vials
10A3	2 vials	5B3	2 vials
7B7	2 vials		

C1 + C4
3/13/98

4E12	1 vial	7B12	1 vial
3C7	1 vial	9B11	1 vial
3G11	1 vial	7E6	1 vial
3H1	1 vial	10B6	1 vial
4E7	1 vial	10A4	1 vial
5G12	1 vial	5A9	1 vial
6A10	1 vial	10B3	1 vial
9C2	1 vial	7G1	1 vial
10A3	1 vial	5B3	1 vial
7B7	1 vial		

HUV-EC-C Human umbilical cord endothelial cells for ALZ

A7- 1 vial 3/2/98

293 Human Embryonic
Kidney cells
Adenovirus Transforme

A7 - 1 vial 3/2/98

!! careful

9/22/98 Slides

1095 - 9-10-98 - treated - mouse that died after 5A11 retreatment
Tg +

1090 - 9/8/98 - Black Mouse 9/4/98
♀ Tg + DOB 6/30/97
DOA 9/16/97

Normal = non-Tg mouse

1072 - 8/11/98 Tg + Normal 2 blocks

9/17/98 - Br Treated 5A11 Tg + D of B 6/30/97

Rem...

IV - acti... this peptide

Boston Biomedical Research Institute
Peptide Synthesis Requisition Form
ABI 431A Synthesizer

17 ml

Please complete the following form and submit it to Anna Wong for each peptide to be synthesized.

Date 3/16/98 Ext 316

Investigator Name: Vic Raso

Department:

Sequence (use 3 letter code):

H_2N- Cys - Tyr - Glu - Val - His - His - Gln - Lys - Leu - Val - Phe - Phe - Ala - Glu - Asp - Val - Gly -COOH

10 -

The Amino Acids in stock are:

Fmoc-L-Ala	Fmoc-Gln(Trt)	Fmoc-L-Leu	Fmoc-L-Ser(tBu)
Fmoc-L-Arg(Pmc)	Fmoc-L-Glu(OtBu)	Fmoc-L-Lys(Boc)	Fmoc-L-Thr(tBu)
Fmoc-Asn(Trt)	Fmoc-Gly	Fmoc-L-Met	Fmoc-L-Trp
Fmoc-L-Asp(OtBu)	Fmoc-L-His(Trt)	Fmoc-L-Phe	Fmoc-L-Tyr(tBu)
Fmoc-L-Cys(Trt)	Fmoc-L-Ile	Fmoc-L-Pro	Fmoc-L-Val)

Choose a Resin: Rink Resin (Amide) ✓ HMP Resin (Acid) ____

Choose a Scale: 0.10mmole ____ (for 20 residue, assuming 80-90% cleavage recovery, yields about 150-175 mg crude peptide)

0.25mmole ✓ (for 20 residue, assuming 80-90% cleavage recovery, yields about 390-~~440~~ mg crude peptide)

Do you want the N-Terminal Fmoc removed? Yes ✓ No ____

Additional Instructions ____

Operators Comments Flow Test OK, Started 4/3/98

Fees: Set-Up Charge $25.00
0.25mmole Scale $12.00/Amino Acid
0.10mmole Scale $10.00/Amino Acid

peptide split ½ a ½

Cleavage of the peptide from the resin is not included in this service!!!

B001675

ABI MODEL 431A VERSION 2.00 Fmoc

SYNTHESIS REPORT

SEQUENCE NAME: V. Raso 17 mer 0.75 scale

RESIN: Rink Resin = 0.42 gm

OPERATOR: A. Wong

RUN EDITOR:

Cy: 1 Rpt: 1 M: dd

Cy: 2 Rpt: 17 M: BADEFG

Cy: 19 Rpt: 1 M: bdc

ADD TIME HOLD CYCLE: 0

RESIN SAMPLES: No

EVENTS LOG:		DATE	TIME	CYCLE	MOD	STEP	SEC REM
Synthesis begun		04/03/98	10:31	1	1	1	1
Barcode read	Gly	04/03/98	10:49	2	2	1	9
Barcode read	Val	04/03/98	11:42	3	2	1	9
Barcode read	Asp	04/03/98	12:36	4	2	1	9
Barcode read	Glu	04/03/98	13:30	5	2	1	9
Barcode read	Ala	04/03/98	14:24	6	2	1	9
Barcode read	Phe	04/03/98	15:19	7	2	1	9
Barcode read	Phe	04/03/98	16:13	8	2	1	9
Barcode read	Val	04/03/98	17:07	9	2	1	9
Barcode read	Leu	04/03/98	18:01	10	2	1	9
Barcode read	Lys	04/03/98	18:56	11	2	1	9
Barcode read	Gln	04/03/98	19:50	12	2	1	9

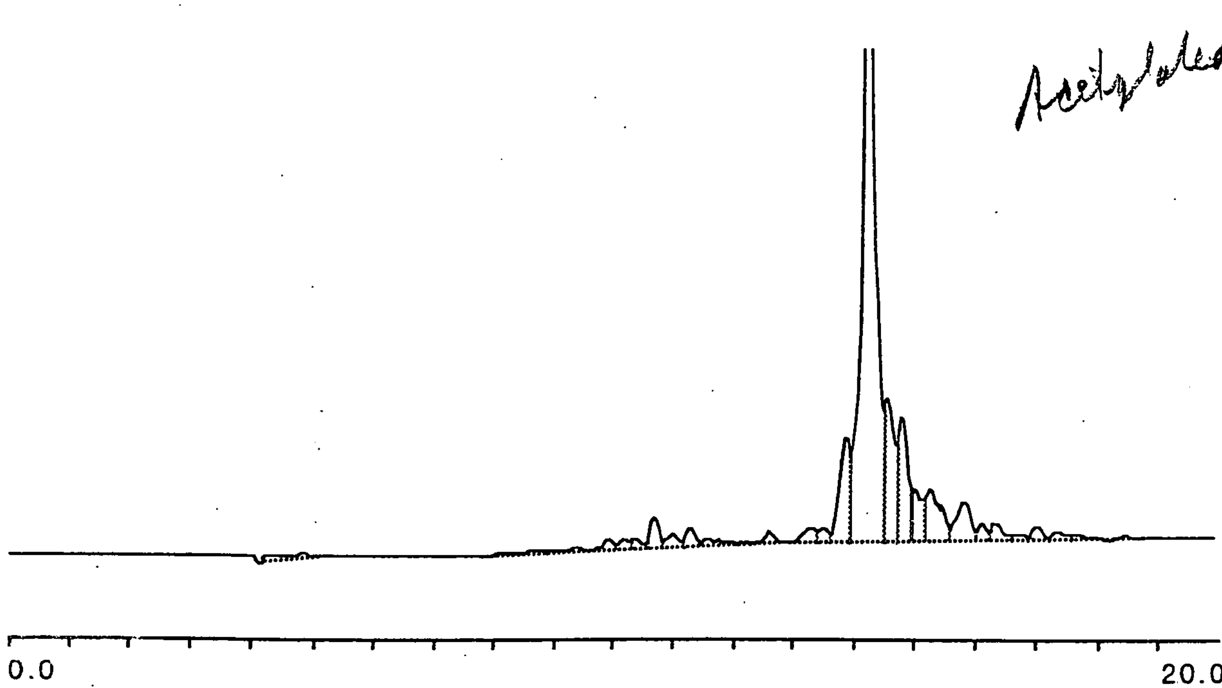
Barcode read	His	04/03/98	20:44	13	2	1	9
Barcode read	His	04/03/98	21:39	14	2	1	9
Barcode read	Val	04/03/98	22:33	15	2	1	9
Barcode read	Glu	04/03/98	23:28	16	2	1	9
Barcode read	Tyr	04/04/98	00:23	17	2	1	9
Barcode read	Cys	04/04/98	01:17	18	2	1	9
Synthesis complete		04/04/98	02:42	19	3	49	0

Date: Thu, Apr 9, 1998 9:25 AM
Data: pepanal 0-100-09APR98-005

Sample: Raso 17-mer acetylated 25µg injected
Column: C5 analytical 1ml/min
Buffer A: 0.1% TFA; Buffer B: 0.1%TFA in 99.9% CH3CN
Gradient: 0-100%B, 20'
Monitor: 220nm, 2.0 AUFS; 280nm, 1.0 AUFS

Processing File: profile#1
Method: pepanal 0-100
Sampling Int: 0.1 Seconds

Data:



Analysis: Channel A

Peak No.	Time	Type	Height(µV)	Area(µV-sec)	Area%
1	4.833	N	5991	301853	1.373
2	8.685	N1	5299	108289	0.492
3	9.921	N4	12235	133552	0.607
4	10.216	N5	12034	121486	0.552
5	10.360	N6	13067	118242	0.538
6	10.668	N7	40475	296837	1.351
7	10.978	N8	15160	194175	0.883
8	11.258	N9	24391	203513	0.926
9	11.550	N10	6751	150316	0.684
10	12.593	N12	13337	101685	0.462

Date: Thu, Apr 9, 1998 9:25 AM
Data: pepanal 0-100-09APR98-005

Analysis: Channel A

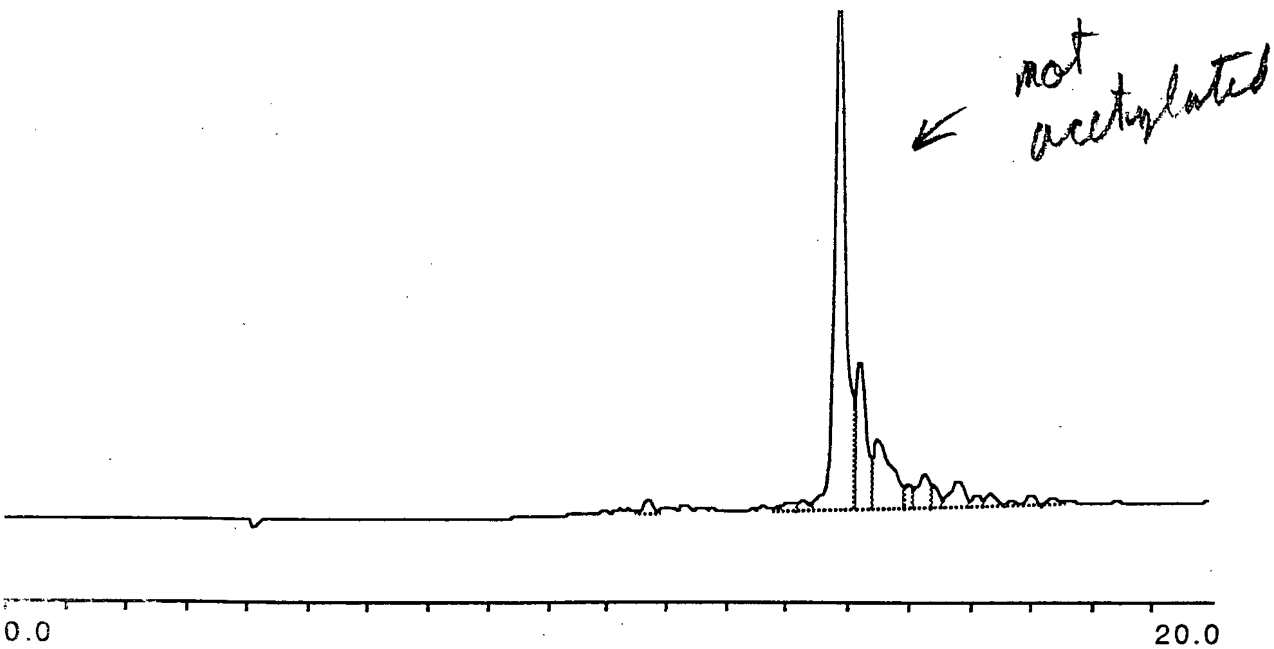
Peak No.	Time	Type	Height(µV)	Area(µV-sec)	Area%
11	13.285	N1	18399	203576	0.926
12	13.483	N2	15886	189637	0.863
13	13.853	N3	135926	1357611	6.179
14	14.181	N4	816316	10999738	50.069
15	14.533	N5	188584	2187892	9.959
16	14.786	N6	161526	1543324	7.025
17	14.975	N7	67265	817543	3.721
18	15.263	N8	67365	1150360	5.236
19	15.835	N9	50784	751768	3.421
20	16.120	N10	20463	197003	0.896
21	16.326	N11	22661	273588	1.245
22	16.696	N12	8780	111649	0.508
23	17.016	N13	18891	183284	0.834
24	17.353	N14	9565	135223	0.615
25	18.431	N15	4161	136780	0.622
Total Area				21968924	99.987

Date: Thu, Apr 9, 1998 8:49 AM
Data: pepanal 0-100-09APR98-004

Sample: Raso 17-mer unacetylated 25µg injected
Column: C5 analytical 1ml/min
Buffer A: 0.1% TFA; Buffer B: 0.1%TFA in 99.9% CH3CN
Gradient: 0-100%B, 20'
Monitor: 220nm, 2.0 AUFS; 280nm, 1.0 AUFS

Processing File: profile#1
Method: pepanal 0-100
Sampling Int: 0.1 Seconds

Data:



Analysis: Channel A

Peak No.	Time	Type	Height(µV)	Area(µV-sec)	Area%
1	10.673	N7	16653	133269	0.975
2	13.088	N3	10678	163364	1.195
3	13.285	N4	11455	133139	0.974
4	13.815	N5	660099	7324481	53.586
5	14.166	N6	188926	1914334	14.005
6	14.466	N7	89294	1888231	13.814
7	14.970	N8	28986	236050	1.726
8	15.241	N9	42523	544355	3.982
9	15.381	N10	29351	253598	1.855
10	15.773	N11	33162	506888	3.708

Date: Thu, Apr 9, 1998 8:49 AM
Data: pepanal 0-100-09APR98-004

Analysis: Channel A

Peak No.	Time	Type	Height(µV)	Area(µV-sec)	Area%
11	16.100	N12	14828	139119	1.017
12	16.306	N13	16033	182751	1.337
13	16.996	N15	13680	131388	0.961
14	17.335	N16	8976	117539	0.859
Total Area				13668506	99.994

SERI/BBRI ANIMAL CARE AND USE COMMITTEE
ANNUAL PROTOCOL UPDATE FORM

Please complete sections A-C.

A. Study Data

Protocol Number: B-027-0800

Principal Investigator: Vic Raso

Title of Protocol: Immunotherapy of Transgenic Alzheimer's mice

Class of Research (circle one): X I II III IV

Original Approval Date: 7/29/97 Last Update: –

Total number of animals used (by species) since 7/29/97 : 25 mice

B. Check all previously approved changes and indicate date(s) of approval.

1. Investigators/Support Staff Change? ___ Yes X No

2. Numbers of Animals Change? ___ Yes X No

3. Species of Animals Change? ___ Yes X No

4. Procedure or Animal Use Change? X Yes ___ No 4/6/98

No amendments to an existing protocol can be made on this form.

C. Investigator's Assurance

I certify that all changes in my animal protocol indicated have been approved by the SERI/BBRI ACUC. Also, I certify that the research proposed in these continuation studies does not duplicate, unnecessarily, other research.

Victor Raso 7/14/98

PRINCIPAL INVESTIGATOR DATE

ACUC Use Only-Do not write below this line.

SCHEPENS/SERI ACUC

Protocol Update Status: Approved___ Approved Conditionally___ (see attached)

APPROVED
SERI/BBRI ACUC
JUL 14 1998

IACUC Approval Signature (Date)

APPROVED BY [signature]
APPROVAL DATE 7-15-98
PROTOCOL #

B001745

revised 8/96

SERI/BBRI ANIMAL CARE AND USE COMMITTEE
PROTOCOL AMENDMENT REQUEST FORM

Date [redacted] 4/6/98

Principal Investigator__Vic Raso Victor Raso

(Signature)

Protocol #_B 027-0800

Primary Working Investigator Christine Kearney Christine Kearney

(Signature)

Protocol Section(s) to be amended	Requested Variation(s) & Rationale **(Explain in Detail)** (use additional sheet if necessary)

III. Animal Characteristics: *The investigator should state the required number of animals to be used in the research project.*

Tg 2576 mice (10-50 mice/yr depending on breeding success) which have been breed at our animal facility.

IV. Species Justification:

The justification is the same as described in the original protocol.

VII. Procedures: This section must clearly reflect how the purpose of the study will be approached.

I propose to add 0.1% KI to the drinking water of the experimental mice to test its effect on the biodistribution ^{125}I-labeled peptides or antibodies (0.1μCi). This procedure establishes a thyroid blockade which can improve the specific localization of the radioactive probe. The KI will be added to labeled water bottles of mice residing in the animal facility or in the laboratory three days prior to the start of the experiment. Those animals will then be removed to the investigator's laboratory for treatment with the ^{125}I-probe and for subsequent monitoring as previously described. If adding the KI has no effect on the results, the protocol will be discontinued.

Reference Lan, et al J. Neuro Imaging V6, No 2, 1996 (pp 131-135)

The proposed amendment has [redacted] been approved. If not accepted, no animal activities can be initiated without responding to the issue(s) below.

The amendment was not accepted for the following reason(s):

[ ] Too many proposed changes. Requires writing a new protocol.

[ ] One or more changes in procedure are significant under USDA criteria. Requires writing a new protocol.

[ ] Points above require clarification. Resubmit with additional clarifying information.

[ ] Other:

B001746

ACUC Chair or Veterinarian Robert E Hopper Date 4/7/98

SERI/BBRI ANIMAL CARE AND USE COMMITTEE
PROTOCOL AMENDMENT REQUEST FORM

Date_Feb 25, 1998________________
Principal Investigator__Vic Raso________ Vic Raso
(Signature)

Protocol #_B 027-0800__________
Primary Working Investigator Christine Kearney Christine Kearney
(Signature)

APPROVED
SERI/BBRI ACUC

Protocol Section(s) to be amended — Requested Variation(s) & Rationale **(Explain in Detail)** (use additional sheet if necessary)

III. Animal Characteristics: *The investigator should state the required number of animals to be used in the research project.*

Tg 2576 mice (10-50 mice/yr depending on breeding success) which have been breed at our animal facility.

IV. Species Justification:

The justification is the same as described in the original protocol.

VII. Procedures: This section must clearly reflect how the purpose of the study will be approached.

The Tg 2576 mice will be injected ip or iv with ^{125}I-labeled peptides or antibodies (0.1μCi) and will be kept in disposable cages in the investigators laboratory. Before imaging, the mice will be immobilized by injection ip with 0.15ml of ketamine+xylazine (200mg/kg, 10mg/kg). Animals will be imaged on a molecular imager system at 1h, 24h, and 48h post injection. At the end of the 48h experiment the mice will be euthanized by CO_2 inhalation and stored frozen before disposal as radioactive waste (Harvard Environmental Health and Safety).

The proposed amendment has/has not been approved. If not accepted, no animal activities can be initiated without responding to the issue(s) below.

REH

The amendment was ~~not~~ accepted for the following reason(s):

[ ] Too many proposed changes. Requires writing a new protocol.

[ ] One or more changes in procedure are significant under USDA criteria. Requires writing a new protocol.

[ ] Points above require clarification. Resubmit with additional clarifying information.

SERI/BBRI ACUC
APPROVED

B001748

[ ] Other:

ACUC Chair or Veterinarian Robert E. Hopkins Date 2/25/98

ANIMAL CARE AND USE SERI/BBRI COMMITTEE
ANNUAL PROTOCOL UPDATE FORM

Please complete sections A-C.

A. Study Data Protocol Number: B-046-0401

Principal Investigator: Vic Raso, Ph.D.

Title of Protocol: Production of Monoclonal Antibodies for Treating AIDS and Alzheimer's Disease

Class of Research (circle one I II III IV

Original Approval Date: 4/27/98 Last Update: ______

Total **number** of animals used (by species) since: 4/27/98 : 100

B. Check all previously approved changes and indicate date(s) of approval.

1. Investigators/Support Staff Change? ____Yes X No

2. Numbers of Animals Change? ____Yes X No

3. Species of Animals Change? ____Yes X No Rabbit

4. Procedure or Animal Use Change? ____Yes X No

No amendments to an existing protocol can be made on this form.

C. Investigator's Assurance

I certify that all changes in my animal protocol indicated have been approved by the SERI/BBRI ACUC. Also, I certify that the research proposed in these continuation studies does not duplicate, unnecessarily, other research.

Vic Raso
PRINCIPAL INVESTIGATOR

3/11/99
DATE

ACUC Use Only-Do not write below this line

SCHEPENS/BBRI ACUC

Protocol Update Status: Approved ______ Approved Conditionally ______ (see attached)

APPROVED
SERI/BBRI
MAR 11 1999

IACUC Approval Signature

APPROVED BY [signature] Date

APPROVAL DATE 3-12-99

PROTOCOL # ______

revised 8/96

B001751

SCHEPENS/BBRI ANIMAL CARE AND USE COMMITTEE

20 Staniford Street Boston, MA 02114

DATE: 5/4/98

TO: Dr. V. Raso

FROM: Andrew Taylor, Ph.D

RE: Production of Monoclonal Antibodies for Treating AIDS and Alzheimer's Disease

Your ACU protocol titled above was reviewed by the ACUC on 3/18/98 and is **approved.** For ordering and future reference, this protocol has been assigned:

Protocol Number:
B-046-0401

Approval Date:
4/27/98

Thank you,

Kathy Murphy
Andrew Taylor, Ph.D.,
Chair, SERI/BBRI ACUC

(Revised 4/1/97)

SCHEPENS/BBRI ANIMAL CARE AND USE PROTOCOL FORM

(Please Read Directions Before Filling Out Form)

ACUC USE ONLY

New Protocol #   Date of Approval

Expiration Date of New Protocol

Old Related Protocol #(s   Other Principal Investigators

SECTION A

Application Data

Title of Protocol (Not grant title, must indicate procedure) Production of Monoclonal Antibodies for Treating AIDS and Alzheimer's Disease

Principal Investigator Vic Raso

Primary Working Investigator(s) Vic Raso,

Technician(s)/Student(s) Christine Kearney

Anticipated Starting Date 3/26/98   **SERI/BBRI Telephone Ext. #** 316

Emergency Contact Person & Telephone # Vic Raso, 734-1405

Backup Emergency Contact Person & Telephone # Christine Kearney 891-7263

2. **Class of Research:** check the appropriate class of experimentation.

_______Class I.   ______XClass II.   ______Class III.   ______Class IV.

______ Tissue Collection Only

3. **Animals**

Species/Strain BALB/c mice Sex Both Age/Weight 1-18 months Source/ Vendor Bred In House Location of Housing Animal Facility #Animals per Experiment ~5 #/Animals per year ~100

4. **Will the animals need special care e.g. special housing, diet, handling, drug treatment, health checks, etc. ? [X] No [ ] Yes, describe.**

5. Will the animals be inoculated with biological products (i.e. tumor line, cultured cells, supernatants, etc.?) [ ] No [X] Yes, describe the source/origin Synthetic peptide antigens synthesized at BBRI. Hybridoma cells developed at BBRI.

6. Biohazardous Material.

Infectious agents   Carcinogens   Radioisotopes   Recombinant DNA   Toxic Substances   Other

Other Synthetic peptide antigens synthesized at BBRI which are unlikely to be biohazardous but extensive testing has not been performed on these substances so they will be assumed harzardous.

SECTION B

The SERI/BBRI ACUC requires the following information to comply with the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals, the ILAR Guide, and the USDA Animal Welfare Act. Address each item independently, without reliance on information covered in other sections. Do not submit major sections of your grant proposal or excessive details of assays not related to the use of animals (e.g. biochemical and/or molecular biology assays, in vitro tests). Define all abbreviations and terms which are not commonly used.

Since not all reviewers are familiar with your area of research, use laypersons terms when providing the following information.

1. Specific Objectives and Potential Value of the Research. Develop an immunological treatment for Alzheimer's disease and AIDS using monoclonal catalytic antibodies.

2. Dated Assurance that this Study Does Not Duplicate any Previous Studies. An extensive literature search performed during the course of writing several NIH grants (1998) has shown that this study does not duplicate any previous work. Keywords : alzheimer/antibody, HIV/antibody, catalytic/antibody, idiotype/vaccine, catalytic/vaccine, plus various researchers in the field

3. Describe Possible Alternatives to Animal Use. There are no *in vitro* alternatives since the protocol involves actively immunizing mice to obtain spleenocytes for hybridoma production. Large amounts of purified monoclonal antibodies are required so that ascites produced in mice will be the main source. Alternatives to animal use are being actively examined. For example, we have obtained flexible gas permeable tissue culture bags to test for in vitro antibody production.

4. Species Justification. The BALB/c mice are compatible with the myeloma cells used for cell fusion and are histocompatible with the hybrodoma cells used for ascites production.

5. Justify Animal Numbers. Five mice will be used for immunization with each antigen. The actual number of immunizations per year will be ~5/year (~25 mice). Five mice will be used for ascites production with each selected hybridoma clone, ~15/year (~75 mice).

6. Procedures. Animals will be immunized (at 1-2 months old) with human ß-amyloid peptide or HIV peptide antigens by s.c. or i.p. injection in complete Freunds adjuvant (0.2ml). They will be boosted s.q. or i.p. using antigen in incomplete adjuvant and/or i.v. using antigen in saline. The animals will be bled from the tail using a small cut to the tail vein and antibody levels will be measured. Direct pressure will be used to stop bleeding. Typically no anesthesia is used for this procedure and mice are restrained for less then 10 min in a commercial mouse holder. The tail vein is nicked with a scalpel blade (cut ~ 2 mm) and 0.1-0.5 ml blood is collected. The mouse usually stops bleeding on its own but direct pressure can be applied using gauze. Bleeding and i.v. injections will be performed in the laboratory because we have a set-up that includes a secured restrainer, a heating lamp and a binocular microscope which allow us to carry out the procedure with more accuracy and minimum difficulty. The mouse will then be sacrificed using CO_2 and its spleen will be used to create hybridomas.

For ascites production BALB/c mice will be primed by ip injection of 0.2 ml of pristane and then 1 week later will be injected ip with hybridoma cells. When sufficient ascites has accumulated the mice will be tapped using an 18 ga. needle. The mice will be monitored thereafter and tapped approximately once every two days. The mice will be tapped 2-3 times or until they show signs of distress, at which point they will be euthanized.

7. Alternatives to Potentially Painful Procedures. At this time there are no proven in vitro techniques that can fully substitute for all the uses of the ascites method of monoclonal antibody production. However, tecnological, alternatives to animal use are being actively examined. For example, we have obtained flexible gas permeable tissue culture bags to test for in vitro antibody production. We will minimize pain and suffering and will be vigilant in detecting critical clinical signs when using the ascites method.

8. Mechanical Restraints. A mouse holder will be used when the animals are injected i.v. or bled (<10min).

9. Post-Procedure Clinical Signs, Monitoring and Care of Animals. No surgical proceedures will be performed.

10. Method of Euthanasia or Disposition of Animals. The animals will be sacrificed using CO_2.

11. If animals are found dead, are they to be [ ] discarded (investigator will be notified) or [X] saved for investigator (specify method for preserving and labeling). Preferably refrigerate, but they can be stored frozen.

12. Criteria for Premature Euthanasia of Individual Animals. If the animals appeared overly stressed or sick they will be euthanized. This might include poor appearence, failure to eat or drink and erratic or lack of movement.

SECTION C

1. Investigator(s) and Support Staff.

Vic Raso, P.I. > 10years experience handling mice and producing monoclonal antibodies (all aspects, immunization, hybridoma production, ascites production, etc.) and Christine Kearney, Technician, trained at BBRI > 1yr experience handling mice and producing monoclonal antibodies (all aspects, immunization, hybridoma production, ascites production, etc.)

Both of us will perform the studies outlined above.

2. Key References that Support This Study with Dates (5 Maximum).

1. Solomon, B., Koppel, R., Frankel, D., and Hanan-Aharon, E. (1997) Disaggregation of Alzheimer beta-amyloid by site-directed mAb. *Proceedings of the National Academy of Sciences of the United States of America* **94**(8), 4109-12.

2. Solomon, B., Koppel, R., Hanan, E., and Katzav, T. (1996) Monoclonal antibodies inhibit in vitro fibrillar aggregation of the Alzheimer beta-amyloid peptide. *Proceedings of the National Academy of Sciences of the United States of America* **93**(1), 452-5.

3. Anticipated Funding Source. PHS NIH grant submitted

4. Status of Grant Application. NIH Application submitted

SECTION D

Certification of Principal Investigator.

Signature certifies that the principal investigator will conduct the project in full compliance with the PHS Policy on Humane Care and Use of Laboratory Animals, the ILAR Guide, the USDA Animal Welfare Act and Schepens Eye Research Institute and Boston Biomedical Research Institute policies governing the use of live vertebrate animals for research purposes. It is understood that ACUC approval is valid for a **maximum period of 36 months following the date of original approval.** Annual updates are required. <u>If minor changes to the original approved protocol are needed, an Amendment Form requesting the changes must</u>

be submitted to and approved by the ACUC chairperson or Veterinarian. This includes changes of personnel named in the original protocol. If the protocol is to be discontinued, notice of this should be sent to the ACUC.

The ACUC requires one (1) **original and** fifteen (15) **copies** in order to process the proposal for full committee review. **Insufficient information will result in return of the protocol for completion and could delay the review process.** Research on animals submitted in this protocol may not begin until the Schepens/BBRI ACUC has **reviewed and approved** the proposal.

4/22/98 Sr. Sci.

Signature **Date** **Position**

SCHEPENS/BBRI ACUC
APPROVED
SER/BBRI ACUC
APR 27 1998
APPROVED BY
APPROVAL DATE 4-27-98
PROTOCOL #

B001757

SCHEPENS/BBRI ANIMAL CARE AND USE COMMITTEE

20 Staniford Street Boston, MA 02114

DATE: March 24, 1998

TO: Vic Raso, Ph.D

FROM: Andrew Taylor, ACUC Chair

RE: Protocol entitled "Production of Monoclonal Antibodies for Treating AIDS and Alzheimer's Disease".

Your ACU protocol received on February 26, 1998 was reviewed by the ACUC on March 18, 1998 and was **approved with revisions**. The following clarifications must be addressed in order that approval be granted. Please feel free to consult me if you have any questions over the following points. Unfortunately, because of regulations specified under the Animal Welfare Act, no animal use under these protocols can be conducted until the protocol is approved. Please return an **original of the revised protocol within one month.**

****It is expected that these procedures will be done in the Animal Facility. If not, please justify removing mice from the facility.**

1. **p.1,#2A. Class of Research**
   Please change class indicated from Class IV to Class II.

2. **p.3, #6B. Procedures**
   Please replace s.q. with s.c.
   Please indicate you will use direct pressure to stop bleeding.
   0.2 is the appropriate dose of pristane.
   How many times or how long will you be tapping mice? When will you stop and euthanize mice.

3. **p.3,#7B. Alternatives to potentially painful procedures.**
   Please use attached statement to formulate your justification.

4. **p.3,#11B. If animals are found dead, are they to be discarded?**
   Can animals be frozen/ i.e. stored at -20°C storage in the animal facility?

Page 2, Dr. Raso
ACUC Protocol Review
March 23, 1998

5. p.3,#12B. **Criteria for Premature Euthanasia of Individual Animals.**
Please state criteria used to determine stress.

Sincerely,

Andrew Taylor

Andrew Taylor, Ph.D
Chair ACU Committee

STRAIN	RATIO	FEMALE DOB	MALE DOB	CAGE ID	DATE MATED	COMMENTS
(B6SJL)F1 x Tg 2576	1:1	5/14/97	6/2/97		10/1/97	Male removed from breeder until otherwise notified
(B6SJL)F1 x Tg 2576	1:6	10/31-11/2/97	1/12/98		2/26/98	Male removed from breeder until otherwise notified
Tg 2576 x (B6SJL)F1	1:1	12/29/97	10/31-11/2/98		2/26/98	Male removed from breeder until otherwise notified

Tg 2576 mice

STRAIN	RATIO	FEMALE DOB	MALE DOB	CAGE ID	DATE MATED	COMMENTS
(B6SJL)F1	6	10/31-11/2/97			2/26/98	Male removed from cage, to Raso 8/23/98.
(B6SJL)F1	1		10/31-11/2/98		2/26/98	Male removed from cage. Tg female to Raso 8/23/98

STRAIN	ROOM #	BC#	RATIO	FEMALE DOB	MALE DOB	CAGE ID	DATE MATED	COMMENTS
(B6SJL)F1			0	2/29/97			2/26/98	Male removed from cage, to Raso 8/23/98. Females sac'd on 12/22/98.
B6SJLF1xTg2576	069		1:2	5/3/99	6/15/99		6/23/99	In room 070. Male#AA95
B6SJLF1xTg2576	069		1:2	5/3/99	6/27/88		6/23/99	In room 070. Male#AA98

B001762

Tg 2576 mice 9/14/98

STRAIN	DOB	SEX	# OF MICE	CAGE ID	MOUSE # POSITIVE	COMMENTS
(B6SJL)F1 Tg 2576	3/31/98	MALE	1	AA-24	(+)	
(B6SJL)F1 Tg 2576	6/9/98	MALE	1	AA-85	(+)	
(B6SJL)F1 Tg 2576	6/9/98	FEMALE	2	AA-80	(+)	
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-91	(+)	
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-92	(+)	9/12/98 wh, bk = Alz-KLH 9/13/98 br = KLH control
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	2	AA-93	(+)	sick inner ear
Tg2576 (B6SJL)F1	3/17-20/98	MALE	1	AA-18	(+)	
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-49	(+)	
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-50	(+)	
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-51	(+)	9/12/98 = Alz-KLH
Tg2576 (B6SJL)F1	5/1/98	FEMALE	1	AA-53	(+)	9/13/98 = KLH control
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/12/98 = Alz-KLH
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/13/98 = KLH control
	3/30/98	FEMALE	4	AA-22	1(+) 3(-)	

Tg 2576 mice 9/14/98

	3/18/98	FEMALE	2	AA-20	(+)	
	3/17-20/98	FEMALE	2	AA-13	(+)	
	5/14/97	MALE	1		(+)	
	8/13/97	FEMALE	4		2(+) 2(-)	
	6/30/97	MALE	1		(+)	
	10/31-11/2/97	MALE	1		(+)	
	10/31-11/2/97	FEMALE	1		(+)	
	5/14/97	FEMALE	3		(+)	DOA 9/16/97 2bk got 5A11 1 died 1br control
	6/30/97	FEMALE	3		(+)	DOA 9/16/97 1br got 5A11 1bk control sac for brain 1br control

B001764

Tg 2576 mice 9/28/98

STRAIN	DOB	SEX	# OF MICE	CAGE ID	MOUSE # POSITIVE	COMMENTS
(B6SJL)F1 Tg 2576	3/31/98	MALE	1	AA-24	(+)	
(B6SJL)F1 Tg 2576	6/9/98	MALE	1	AA-85	(+)	
(B6SJL)F1 Tg 2576	6/9/98	FEMALE	2	AA-80	(+)	
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-91	(+)	
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-92	(+)	9/12/98 wh, bk = Alz-KLH 9/13/98 br = KLH control
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	2	AA-93	(+)	sick inner ear
Tg2576 (B6SJL)F1	3/17-20/98	MALE	1	AA-18		
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-49		
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-50	(+)	
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-51	(+)	9/12/98 = Alz-KLH
Tg2576 (B6SJL)F1	5/1/98	FEMALE	1	AA-53	(+)	9/13/98 = KLH control
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/12/98 = Alz-KLH
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/13/98 = KLH control
5A11 Exp/OLD Imm	5/14/97	FEMALE	3		(+)	DOA 9/16/97 2Bk= 5A11 1Br= Control then got Alz-KLH 9/28/98
5A11 Exp/OLD Imm	6/30/97	FEMALE	3		(+)	DOA 9/16/97 Br=5A11 2Bk= control then one kill other got Alz-KLH 9/28/98

	3/30/98	FEMALE	4	AA-22	3neg 1pos	
	3/17-20/98	FEMALE	2	AA-13	(+)	
	3/18/98	FEMALE	2	AA-20	(+)	
	5/14/97	MALE	1		(+)	
	8/13/97	FEMALE	4		2neg 2pos	
	6/30/97	MALE	1		(+)	
	10/31-11/2/97	MALE	1		(+)	
	10/31-11/2/97	FEMALE	1		(+)	

Tg 2576 mice 10/24/98

STRAIN	DOB	SEX	# OF MICE	CAGE ID	MOUSE # POSITIVE	COMMENTS
(B6SJL)F1 Tg 2576	3/31/98	MALE	1	AA-24	(+)	
(B6SJL)F1 Tg 2576	6/9/98	MALE	1	AA-85	(+)	
(B6SJL)F1 Tg 2576	6/9/98	FEMALE	2	AA-80	(+)	
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-91	(+)	
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-92	(+)	9/12/98 wh, bk = Alz-KLH/CFA 10/24/98 same IFA 9/13/98 br = KLH control 10/24/98 same IFA
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	2	AA-93	(+)	sick inner ear ??
Tg2576 (B6SJL)F1	3/17-20/98	MALE	1	AA-18	(+)	
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-49	(+)	9/12/98 = Alz-KLH (was written on cage but not in cell) 10/24/98 same IFA
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-50	(+)	9/13/98 = KLH control (this cell was not marked but cage was marked) 10/24/98 same IFA
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-51	(+)	9/12/98 = Alz-KLH
Tg2576 (B6SJL)F1	5/1/98	FEMALE	1	AA-53	(+)	(this cell had 9/13/98 = KLH control but cage was not marked; cage AA-50 was labeled instead)
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/12/98 = Alz-KLH got same in IFA 10/24/98
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/13/98 =KLH control 10/24/98 same IFA
5A11 Exp/OLD Imm	5/14/97	FEMALE	3		(+)	DOA 9/16/97 2Bk= 5A11 1Br= Control then got Alz-KLH 9/28/98 got same in IFA 10/24/98
5A11 Exp/OLD Imm	6/30/97	FEMALE	3		(+)	DOA 9/16/97 1Br=5A11 1Br= control 1Bk= control then one kill other Br got Alz-KLH 9/28/98 got same in IFA 10/24/98 and died a few min later anaphy? rough handling?
	3/30/98	FEMALE	4	AA-22	3neg 1pos	
	3/17-20/98	FEMALE	2	AA-13	(+)	
	3/18/98	FEMALE	2	AA-20	(+)	
	5/14/97	MALE	1		(+)	
	8/13/97	FEMALE	4		2neg 2pos	
	6/30/97	MALE	1		(+)	
	10/31-11/2/97	MALE	1		(+)	
	10/31-11/2/97	FEMALE	1		(+)	

B001767

STRAIN	DOB	SEX	# OF MICE	CAGE ID	MOUSE # POSITIVE	COMMENTS
(B6SJL)F1 Tg 2576	3/31/98	MALE	1	AA-24	(+)	
(B6SJL)F1 Tg 2576	6/9/98	MALE	1	AA-85	(+)	
(B6SJL)F1 Tg 2576	6/9/98	FEMALE	2	AA-80	(+)	
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-91	(+)	
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-92	(+)	9/12/98 wh, bk = Alz-KLH/CFA 10/24/98 same IFA 9/13/98 br = KLH control 10/24/98 same IFA
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	2	AA-93	(+)	sick inner ear ??
Tg2576 (B6SJL)F1	3/17-20/98	MALE	1	AA-18	(+)	sick killed 12/10/99
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-49	(+)	9/12/98 = Alz-KLH (was written on cage but not in cell) 10/24/98 same IFA
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-50	(+)	9/13/98 = KLH control (this cell was not marked but cage was marked) 10/24/98 same IFA
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-51	(+)	9/12/98 = Alz-KLH
Tg2576 (B6SJL)F1	5/1/98	FEMALE	1	AA-53	(+)	(this cell had 9/13/98 = KLH control but cage was not marked; cage AA-50 was labeled instead)
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/12/98 = Alz-KLH got same in IFA 10/24/98
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/13/98 =KLH control 10/24/98 same IFA
5A11 Exp/OLD Imm	5/14/97	FEMALE	3		(+)	DOA 9/16/97 2Bk= 5A11 1Br= Control then got Alz-KLH 9/28/98 got same in IFA 10/24/98
5A11 Exp/OLD Imm	6/30/97	FEMALE	3		(+)	DOA 9/16/97 1Br=5A11 1Br= control 1Bk= control then one kill other Br got Alz-KLH 9/28/98 got same in IFA 10/24/98 and died a few min later anaphy? rough handling?
	3/30/98	FEMALE	4	AA-22	3neg 1pos	1 sick killed 12/10/99
	3/17-20/98	FEMALE	2	AA-13	(+)	
	3/18/98	FEMALE	2	AA-20	(+)	
	5/14/97	MALE	1		(+)	
	8/13/97	FEMALE	4		2neg 2pos	
	6/30/97	MALE	1		(+)	
	10/31-11/2/97	MALE	1		(+)	10/5/99 removed brain
	10/31-11/2/97	FEMALE	1		(+)	

B001768

STRAIN	ROOM #	BC#	RATIO	FEMALE DOB	MALE DOB	CAGE ID	DATE MATED	COMMENTS
(B6SJL)F1			0	2/29/97			2/26/98	Male removed from cage, to Raso 8/23/98. Females sac'd on 12/22/98.
B6SJLF1xTg2576	069		1:2	5/3/99	6/15/99		6/23/99	In room 070. Male#AA95
B6SJLF1xTg2576	069		1:2	5/3/99	6/27/88		6/23/99	In room 070. Male#AA98

B001769

Tg 2576 mice 2

STRAIN	DOB	SEX	# OF MICE	CAGE ID	MOUSE # POSITIVE	COMMENTS
(B6SJL)F1 Tg 2576	3/31/98	MALE	1	AA-24	(+)	
(B6SJL)F1 Tg 2576	6/9/98	MALE	1	AA-85	(+)	
(B6SJL)F1 Tg 2576	6/9/98	FEMALE	2	AA-80	(+)	
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-91	(+)	
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-92	(+)	9/12/98 wh, bk = Alz-KLH 9/13/98 br = KLH control
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	2	AA-93	(+)	sick inner ear
Tg2576 (B6SJL)F1	3/17-20/98	MALE	1	AA-18		
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-49		
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-50	(+)	
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-51	(+)	9/12/98 = Alz-KLH
Tg2576 (B6SJL)F1	5/1/98	FEMALE	1	AA-53	(+)	9/13/98 = KLH control
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/12/98 = Alz-KLH
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/13/98 = KLH control

B001770

Tg 2576 mice

STRAIN	DOB	SEX	# OF MICE	CAGE ID	MOUSE # POSITIVE	COMMENTS
(B6SJL)F1 Tg 2576	6/15/98	MALE	1	AA-95	(+)	
(B6SJL)F1 Tg 2576	6/15/98	MALE	1	AA-95	(+)	
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-91	(+)	
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-49	(+)	
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-50	(+)	
Tg2576 (B6SJL)F1	6/27/98	MALE	1	AA-98	(+)	
Tg2576 (B6SJL)F1	6/27/98	MALE	1	AA-99	(+)	
Tg2576 (B6SJL)F1	6/27/98	FEMALE	1	AA-97	(+)	

B001771

Tg 2576 mice

STRAIN	RATIO	FEMALE DOB	MALE DOB	CAGE ID	DATE MATED	COMMENTS
(B6SJL)F1	6	10/31-11/2/97			2/26/98	Male removed from cage, to Raso 8/23/98.
(B6SJL)F1	1		10/31-11/2/98		2/26/98	Male removed from cage. Tg female to Raso 8/23/98

B001772

STRAIN	BC #	ROOM #	DOB	SEX	# OF MICE	CAGE ID	MOUSE # POSITIVE	COMMENTS
B6(SJL)F1	JAX		10/31-11/2/98	MALE	1			removed from breeder Removed from Colony. Sac'd 6/23/99
B6(SJL)F1	JAX	069	5/3/99	FEMALE	1			
(B6SJL)F1 Tg 2576		068	6/15/98	MALE	1	AA-95	(+)	Moved to '068
(B6SJL)F1 Tg 2576		001	6/15/98	FEMALE	3	AA-91	(+)	Moved to room 001 on 6/99
(B6SJL)F1 Tg 2576		001	6/27/98	FEMALE	1	AA-97	(+)	Moved to room '001
(B6SJL)F1 Tg 2576	119	001	7/14/99	MALE	3	AD-11	all neg (-)	Moved to room '001
(B6SJL)F1 Tg 2576	119	001	7/14/99	FEMALE	3	AD-12	all neg (-)	Moved to room '001
(B6SJL)F1 Tg 2576		068	7/17-18/99	MALE	5	AD-13	#1, 4, (+)	#1 found dead 9/20/99
(B6SJL)F1 Tg 2576		068	7/17-18/99	MALE	5	AD-13	# 5 (+)	
(B6SJL)F1 Tg 2576		001	7/17-18/99	MALE	5	AD-13	#2, 3 (-)	Moved to room '001
(B6SJL)F1 Tg 2576		001	7/17-18/99	MALE	2	AD-14	all pos (+)	Moved to room '001
(B6SJL)F1 Tg 2576		001	7/17-18/99	FEMALE	5	AD-15	#4 pos (+)	Moved to room '001
(B6SJL)F1 Tg 2576		001	7/17-18/99	FEMALE	3	AD-16	all pos (+)	Moved to room '001
(B6SJL)F1 Tg 2576		001	7/17-18/99	FEMALE	3	AD-17	#1 pos (+)	Moved to room '001
(B6SJL)F1 Tg 2576	119	068	8/23/99	MALE	5			
(B6SJL)F1 Tg 2576	119	068	8/23/99	MALE	5			
(B6SJL)F1 Tg 2576	119	068	8/23/99	FEMALE	5			
(B6SJL)F1 Tg 2576	119	068	8/23/99	FEMALE	4			
(B6SJL)F1 Tg 2576	120	068	9/27/99	MALE	4			
(B6SJL)F1 Tg 2576	120	068	9/27/99	FEMALE	3			
(B6SJL)F1 Tg 2576	120	068	9/27/99	FEMALE	3			

B001773

Tg 2576 Raso's mice

STRAIN	BC #	ROOM #	DOB	SEX	# OF MICE	CAGE ID	MOUSE # POSITIVE	COMMENTS
B6(SJL)F1	JAX		10/31-11/2/98	MALE	1			removed from breeder Removed from Colony. Sac'd 6/23/99
B6(SJL)F1	JAX	069	5/3/99	FEMALE	1			
(B6SJL)F1 Tg 2576		068	6/15/98	MALE	1	AA-95	(+)	Moved to '068
(B6SJL)F1 Tg 2576			6/15/98	FEMALE	3	AA-91	(+)	Moved to room 001 on 6/99
(B6SJL)F1 Tg 2576			6/27/98	FEMALE	1	AA-97	(+)	Moved to room '001
(B6SJL)F1 Tg 2576	119	068	7/14/99	MALE	3	AD-11	all neg (-)	
(B6SJL)F1 Tg 2576	119	068	7/14/99	FEMALE	3	AD-12	all neg (-)	
(B6SJL)F1 Tg 2576		068	7/17-18/99	MALE	5	AD-13	#1, 4, 5 (+)	
(B6SJL)F1 Tg 2576		068	7/17-18/99	MALE	2	AD-14	all pos (+)	1 mouse found dead 9/1/99
(B6SJL)F1 Tg 2576		068	7/17-18/99	FEMALE	5	AD-15	#4 pos (+)	
(B6SJL)F1 Tg 2576		068	7/17-18/99	FEMALE	3	AD-16	all pos (+)	
(B6SJL)F1 Tg 2576		068	7/17-18/99	FEMALE	3	AD-17	#1 pos (+)	
(B6SJL)F1 Tg 2576	119	068	8/23/99	MALE	5			
(B6SJL)F1 Tg 2576	119	068	8/23/99	MALE	5			
(B6SJL)F1 Tg 2576	119	068	8/23/99	FEMALE	5			
(B6SJL)F1 Tg 2576	119	068	8/23/99	FEMALE	4			

B001775

Tg 2576 mice 11/18/98

STRAIN	DOB	SEX	# OF MICE	CAGE ID	MOUSE # POSITIVE	COMMENTS			
(B6SJL)F1 Tg 2576	3/31/98	MALE	1	AA-24	(+)				
(B6SJL)F1 Tg 2576	6/9/98	MALE	1	AA-85	(+)				
(B6SJL)F1 Tg 2576	6/9/98	FEMALE	2	AA-80	(+)				
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-91	(+)				
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-92	(+)	9/12/98 wh, bk = Alz-KLH/CFA 10/24/98 same IFA 9/13/98 br = KLH control 10/24/98 same IFA			
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	2	AA-93	(+)	sick inner ear ??			
Tg2576 (B6SJL)F1	3/17-20/98	MALE	1	AA-18	(+)				
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-49	(+)	9/12/98 = Alz-KLH (was written on cage but not in cell) 10/24/98 same IFA			
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-50	(+)	9/13/98 = KLH control (this cell was not marked but cage was marked) 10/24/98 same IFA			
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-51	(+)	9/12/98 = Alz-KLH			
Tg2576 (B6SJL)F1	5/1/98	FEMALE	1	AA-53	(+)	(this cell had 9/13/98 = KLH control but cage was not marked; cage AA-50 was labeled instead)			
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/12/98 = Alz-KLH got same in IFA 10/24/98			
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/13/98 =KLH control 10/24/98 same IFA			
5A11 Exp/OLD Imm	5/14/97	FEMALE	3		(+)	DOA 9/16/97 2Bk= 5A11 1Br= Control then got Alz-KLH 9/28/98 got same in IFA 10/24/98			
5A11 Exp/OLD Imm	6/30/97	FEMALE	3		(+)	DOA 9/16/97 1Br=5A11 1Br= control 1Bk= control then one kill other Br got Alz-KLH 9/28/98 got same in IFA 10/24/98 and died a few min later anaphy? rough handling?			
	3/30/98	FEMALE	4	AA-22	3neg 1pos				
	3/17-20/98	FEMALE	2	AA-13	(+)				
	3/18/98	FEMALE	2	AA-20	(+)				
	5/14/97	MALE	1		(+)				
	8/13/97	FEMALE	4		2neg 2pos				
	6/30/97	MALE	1		(+)				
	10/31-11/2/97	MALE	1		(+)				
	10/31-11/2/97	FEMALE	1		(+)				
Tg2576 (B6SJL)F1	6/27/98	MALE	1	AA-99	(+)				

B001776

Tg 2576 mice 10/24/98 rev

STRAIN	DOB	SEX	# OF MICE	CAGE ID	MOUSE # POSITIVE	COMMENTS
(B6SJL)F1 Tg 2576	3/31/98	MALE	1	AA-24	(+)	
(B6SJL)F1 Tg 2576	6/9/98	MALE	1	AA-85	(+)	
(B6SJL)F1 Tg 2576	6/9/98	FEMALE	2	AA-80	(+)	
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-91	(+)	
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-92	(+)	9/12/98 wh, bk = Alz-KLH/CFA 10/24/98 same IFA 9/13/98 br = KLH control 10/24/98 same IFA
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	2	AA-93	(+)	sick inner ear ??
Tg2576 (B6SJL)F1	3/17-20/98	MALE	1	AA-18	(+)	sick killed 12/10/99
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-49	(+)	9/12/98 = Alz-KLH (was written on cage but not in cell) 10/24/98 same IFA
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-50	(+)	9/13/98 = KLH control (this cell was not marked but cage was marked) 10/24/98 same IFA
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-51	(+)	9/12/98 = Alz-KLH
Tg2576 (B6SJL)F1	5/1/98	FEMALE	1	AA-53	(+)	(this cell had 9/13/98 = KLH control but cage was not marked; cage AA-50 was labeled instead)
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/12/98 = Alz-KLH got same in IFA 10/24/98
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/13/98 =KLH control 10/24/98 same IFA
5A11 Exp/OLD Imm	5/14/97	FEMALE	3		(+)	DOA 9/16/97 2Bk= 5A11 1Br= Control then got Alz-KLH 9/28/98 got same in IFA 10/24/98
5A11 Exp/OLD Imm	6/30/97	FEMALE	3		(+)	DOA 9/16/97 1Br=5A11 1Br= control 1Bk= control then one kill other Br got Alz-KLH 9/28/98 got same in IFA 10/24/98 and died a few min later anaphy? rough handling?
	3/30/98	FEMALE	4	AA-22	3neg 1pos	1 sick killed 12/10/99
	3/17-20/98	FEMALE	2	AA-13	(+)	
	3/18/98	FEMALE	2	AA-20	(+)	
	5/14/97	MALE	1		(+)	
	8/13/97	FEMALE	4		2neg 2pos	
	6/30/97	MALE	1		(+)	
	10/31-11/2/97	MALE	1		(+)	10/5/99 removed brain
	10/31-11/2/97	FEMALE	1		(+)	

B001777

Tg 2576 mice 11/18/98

STRAIN	DOB	SEX	# OF MICE	CAGE ID	MOUSE # POSITIVE	COMMENTS			
(B6SJL)F1 Tg 2576	3/31/98	MALE	1	AA-24	(+)				
(B6SJL)F1 Tg 2576	6/9/98	MALE	1	AA-85	(+)				
(B6SJL)F1 Tg 2576	6/9/98	FEMALE	2	AA-80	(+)				
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-91	(+)				
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-92	(+)	9/12/98 wh, bk = Alz-KLH/CFA 10/24/98 same IFA 9/13/98 br = KLH control 10/24/98 same IFA			
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	2	AA-93	(+)	sick inner ear ??			
Tg2576 (B6SJL)F1	3/17-20/98	MALE	1	AA-18	(+)				
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-49	(+)	9/12/98 = Alz-KLH (was written on cage but not in cell) 10/24/98 same IFA			
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-50	(+)	9/13/98 = KLH control (this cell was not marked but cage was marked) 10/24/98 same IFA			
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-51	(+)	9/12/98 = Alz-KLH			
Tg2576 (B6SJL)F1	5/1/98	FEMALE	1	AA-53	(+)	(this cell had 9/13/98 = KLH control but cage was not marked; cage AA-50 was labeled instead)			
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/12/98 = Alz-KLH got same in IFA 10/24/98			
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/13/98 =KLH control 10/24/98 same IFA			
5A11 Exp/OLD Imm	5/14/97	FEMALE	3		(+)	DOA 9/16/97 2Bk= 5A11 1Br= Control then got Alz-KLH 9/28/98 got same in IFA 10/24/98			
5A11 Exp/OLD Imm	6/30/97	FEMALE	3		(+)	DOA 9/16/97 1Br=5A11 1Br= control 1Bk= control then one kill other Br got Alz-KLH 9/28/98 got same in IFA 10/24/98 and died a few min later anaphy? rough handling?			
	3/30/98	FEMALE	4	AA-22	3neg 1pos				
	3/17-20/98	FEMALE	2	AA-13	(+)				
	3/18/98	FEMALE	2	AA-20	(+)				
	5/14/97	MALE	1		(+)				
	8/13/97	FEMALE	4		2neg 2pos				
	6/30/97	MALE	1		(+)				
	10/31-11/2/97	MALE	1		(+)				
	10/31-11/2/97	FEMALE	1		(+)				
Tg2576 (B6SJL)F1	6/27/98	MALE	1	AA-99	(+)				

Tg 2576 mice 10/24/98

STRAIN	DOB	SEX	# OF MICE	CAGE ID	MOUSE # POSITIVE	COMMENTS
(B6SJL)F1 Tg 2576	3/31/98	MALE	1	AA-24	(+)	
(B6SJL)F1 Tg 2576	6/9/98	MALE	1	AA-85	(+)	
(B6SJL)F1 Tg 2576	6/9/98	FEMALE	2	AA-80	(+)	
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-91	(+)	
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-92	(+)	9/12/98 wh, bk = Alz-KLH/CFA 10/24/98 same IFA 9/13/98 br = KLH control 10/24/98 same IFA
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	2	AA-93	(+)	sick inner ear ??
Tg2576 (B6SJL)F1	3/17-20/98	MALE	1	AA-18	(+)	
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-49	(+)	9/12/98 = Alz-KLH (was written on cage but not in cell) 10/24/98 same IFA
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-50	(+)	9/13/98 = KLH control (this cell was not marked but cage was marked) 10/24/98 same IFA
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-51	(+)	9/12/98 = Alz-KLH
Tg2576 (B6SJL)F1	5/1/98	FEMALE	1	AA-53	(+)	(this cell had 9/13/98 = KLH control but cage was not marked; cage AA-50 was labeled instead)
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/12/98 = Alz-KLH got same in IFA 10/24/98
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/13/98 =KLH control 10/24/98 same IFA
5A11 Exp/OLD Imm	5/14/97	FEMALE	3		(+)	DOA 9/16/97 2Bk= 5A11 1Br= Control then got Alz-KLH 9/28/98 got same in IFA 10/24/98
5A11 Exp/OLD Imm	6/30/97	FEMALE	3		(+)	DOA 9/16/97 1Br=5A11 1Br= control 1Bk= control then one kill other Br got Alz-KLH 9/28/98 got same in IFA 10/24/98 and died a few min later anaphy? rough handling?
	3/30/98	FEMALE	4	AA-22	3neg 1pos	
	3/17-20/98	FEMALE	2	AA-13	(+)	
	3/18/98	FEMALE	2	AA-20	(+)	
	5/14/97	MALE	1		(+)	
	8/13/97	FEMALE	4		2neg 2pos	
	6/30/97	MALE	1		(+)	

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Tg 2576 mice 9/28/98

STRAIN	DOB	SEX	# OF MICE	CAGE ID	MOUSE # POSITIVE	COMMENTS
(B6SJL)F1 Tg 2576	3/31/98	MALE	1	AA-24	(+)	
(B6SJL)F1 Tg 2576	6/9/98	MALE	1	AA-85	(+)	
(B6SJL)F1 Tg 2576	6/9/98	FEMALE	2	AA-80	(+)	
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-91	(+)	
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-92	(+)	9/12/98 wh, bk = Alz-KLH 9/13/98 br = KLH control
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	2	AA-93	(+)	sick inner ear
Tg2576 (B6SJL)F1	3/17-20/98	MALE	1	AA-18		
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-49		
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-50	(+)	
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-51	(+)	9/12/98 = Alz-KLH
Tg2576 (B6SJL)F1	5/1/98	FEMALE	1	AA-53	(+)	9/13/98 = KLH control
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/12/98 = Alz-KLH
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/13/98 = KLH control
5A11 Exp/OLD Imm	5/14/97	FEMALE	3		(+)	DOA 9/16/97 2Bk= 5A11 1Br= Control then got Alz-KLH 9/28/98
5A11 Exp/OLD Imm	6/30/97	FEMALE	3		(+)	DOA 9/16/97 Br=5A11 2Bk= control then one kill other got Alz-KLH 9/28/98

Tg 2576 mice 9/28/98

	3/30/98	FEMALE	4	AA-22	3neg 1pos	
	3/17-20/98	FEMALE	2	AA-13	(+)	
	3/18/98	FEMALE	2	AA-20	(+)	
	5/14/97	MALE	1		(+)	
	8/13/97	FEMALE	4		2neg 2pos	
	6/30/97	MALE	1		(+)	
	10/31-11/2/97	MALE	1		(+)	
	10/31-11/2/97	FEMALE	1		(+)	

Tg 2576 mice 2

STRAIN	DOB	SEX	# OF MICE	CAGE ID	MOUSE # POSITIVE	COMMENTS
(B6SJL)F1 Tg 2576	3/31/98	MALE	1	AA-24	(+)	
(B6SJL)F1 Tg 2576	6/9/98	MALE	1	AA-85	(+)	
(B6SJL)F1 Tg 2576	6/9/98	FEMALE	2	AA-80	(+)	
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-91	(+)	
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-92	(+)	9/12/98 wh, bk = Alz-KLH 9/13/98 br = KLH control
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	2	AA-93	(+)	sick inner ear
Tg2576 (B6SJL)F1	3/17-20/98	MALE	1	AA-18		
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-49		
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-50	(+)	
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-51	(+)	9/12/98 = Alz-KLH
Tg2576 (B6SJL)F1	5/1/98	FEMALE	1	AA-53	(+)	9/13/98 = KLH control
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/12/98 = Alz-KLH
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/13/98 = KLH control

Tg 2576 mice

STRAIN	DOB	SEX	# OF MICE	CAGE ID	MOUSE # POSITIVE	COMMENTS
(B6SJL)F1 Tg 2576	6/15/98	MALE	1	AA-95	(+)	
(B6SJL)F1 Tg 2576	6/15/98	MALE	1	AA-95	(+)	
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-91	(+)	
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-49	(+)	
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-50	(+)	
Tg2576 (B6SJL)F1	6/27/98	MALE	1	AA-98	(+)	
Tg2576 (B6SJL)F1	6/27/98	MALE	1	AA-99	(+)	
Tg2576 (B6SJL)F1	6/27/98	FEMALE	1	AA-97	(+)	

STRAIN	BC #	ROOM #	DOB	SEX	# OF MICE	CAGE ID	MOUSE # POSITIVE	COMMENTS
B6(SJL)F1	JAX		10/31-11/2/98	MALE	1			removed from breeder Removed from Colony. Sac'd 6/23/99
B6(SJL)F1	JAX	069	5/3/99	FEMALE	1			
(B6SJL)F1 Tg 2576		068	6/15/98	MALE	1	AA-95	(+)	Moved to '068
(B6SJL)F1 Tg 2576			6/15/98	FEMALE	3	AA-91	(+)	Moved to room 001 on 6/99
(B6SJL)F1 Tg 2576			6/27/98	FEMALE	1	AA-97	(+)	Moved to room '001
(B6SJL)F1 Tg 2576	119	068	7/14/99	MALE	3	AD-11	all neg (-)	
(B6SJL)F1 Tg 2576	119	068	7/14/99	FEMALE	3	AD-12	all neg (-)	
(B6SJL)F1 Tg 2576		068	7/17-18/99	MALE	5	AD-13	#1, 4, 5 (+)	
(B6SJL)F1 Tg 2576		068	7/17-18/99	MALE	2	AD-14	all pos (+)	1 mouse found dead 9/1/99
(B6SJL)F1 Tg 2576		068	7/17-18/99	FEMALE	5	AD-15	#4 pos (+)	
(B6SJL)F1 Tg 2576		068	7/17-18/99	FEMALE	3	AD-16	all pos (+)	
(B6SJL)F1 Tg 2576		068	7/17-18/99	FEMALE	3	AD-17	#1 pos (+)	
(B6SJL)F1 Tg 2576	119	068	8/23/99	MALE	5			
(B6SJL)F1 Tg 2576	119	068	8/23/99	MALE	5			
(B6SJL)F1 Tg 2576	119	068	8/23/99	FEMALE	5			
(B6SJL)F1 Tg 2576	119	068	8/23/99	FEMALE	4			

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Tg 2576 mice 11/18/98

STRAIN	DOB	SEX	# OF MICE	CAGE ID	MOUSE # POSITIVE	COMMENTS			
(B6SJL)F1 Tg 2576	3/31/98	MALE	1	AA-24	(+)				
(B6SJL)F1 Tg 2576	6/9/98	MALE	1	AA-85	(+)				
(B6SJL)F1 Tg 2576	6/9/98	FEMALE	2	AA-80	(+)				
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-91	(+)				
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-92	(+)	9/12/98 wh, bk = Alz-KLH/CFA 10/24/98 same IFA 9/13/98 br = KLH control 10/24/98 same IFA			
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	2	AA-93	(+)	sick inner ear ??			
Tg2576 (B6SJL)F1	3/17-20/98	MALE	1	AA-18	(+)				
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-49	(+)	9/12/98 = Alz-KLH (was written on cage but not in cell) 10/24/98 same IFA			
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-50	(+)	9/13/98 = KLH control (this cell was not marked but cage was marked) 10/24/98 same IFA			
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-51	(+)	9/12/98 = Alz-KLH			
Tg2576 (B6SJL)F1	5/1/98	FEMALE	1	AA-53	(+)	(this cell had 9/13/98 = KLH control but cage was not marked; cage AA-50 was labeled instead)			
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/12/98 = Alz-KLH got same in IFA 10/24/98			
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/13/98 =KLH control 10/24/98 same IFA			
5A11 Exp/OLD Imm	5/14/97	FEMALE	3		(+)	DOA 9/16/97 2Bk= 5A11 1Br= Control then got Alz-KLH 9/28/98 got same in IFA 10/24/98			
5A11 Exp/OLD Imm	6/30/97	FEMALE	3		(+)	DOA 9/16/97 1Br=5A11 1Br= control 1Bk= control then one kill other Br got Alz-KLH 9/28/98 got same in IFA 10/24/98 and died a few min later anaphy? rough handling?			
	3/30/98	FEMALE	4	AA-22	3neg 1pos				
	3/17-20/98	FEMALE	2	AA-13	(+)				
	3/18/98	FEMALE	2	AA-20	(+)				
	5/14/97	MALE	1		(+)				
	8/13/97	FEMALE	4		2neg 2pos				
	6/30/97	MALE	1		(+)				
	10/31-11/2/97	MALE	1		(+)				
	10/31-11/2/97	FEMALE	1		(+)				
Tg2576 (B6SJL)F1	6/27/98	MALE	1	AA-99	(+)				

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Tg 2576 mice 10/24/98 rev

STRAIN	DOB	SEX	# OF MICE	CAGE ID	MOUSE # POSITIVE	COMMENTS
(B6SJL)F1 Tg 2576	3/31/98	MALE	1	AA-24	(+)	
(B6SJL)F1 Tg 2576	6/9/98	MALE	1	AA-85	(+)	
(B6SJL)F1 Tg 2576	6/9/98	FEMALE	2	AA-80	(+)	
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-91	(+)	
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-92	(+)	9/12/98 wh, bk = Alz-KLH/CFA 10/24/98 same IFA 9/13/98 br = KLH control 10/24/98 same IFA
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	2	AA-93	(+)	sick inner ear ??
Tg2576 (B6SJL)F1	3/17-20/98	MALE	1	AA-18	(+)	sick killed 12/10/99
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-49	(+)	9/12/98 = Alz-KLH (was written on cage but not in cell) 10/24/98 same IFA
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-50	(+)	9/13/98 = KLH control (this cell was not marked but cage was marked) 10/24/98 same IFA
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-51	(+)	9/12/98 = Alz-KLH
Tg2576 (B6SJL)F1	5/1/98	FEMALE	1	AA-53	(+)	(this cell had 9/13/98 = KLH control but cage was not marked; cage AA-50 was labeled instead)
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/12/98 = Alz-KLH got same in IFA 10/24/98
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/13/98 =KLH control 10/24/98 same IFA
5A11 Exp/OLD Imm	5/14/97	FEMALE	3		(+)	DOA 9/16/97 2Bk= 5A11 1Br= Control then got Alz-KLH 9/28/98 got same in IFA 10/24/98
5A11 Exp/OLD Imm	6/30/97	FEMALE	3		(+)	DOA 9/16/97 1Br=5A11 1Br= control 1Bk= control then one kill other Br got Alz-KLH 9/28/98 got same in IFA 10/24/98 and died a few min later anaphy? rough handling?
	3/30/98	FEMALE	4	AA-22	3neg 1pos	1 sick killed 12/10/99
	3/17-20/98	FEMALE	2	AA-13	(+)	
	3/18/98	FEMALE	2	AA-20	(+)	
	5/14/97	MALE	1		(+)	
	8/13/97	FEMALE	4		2neg 2pos	
	6/30/97	MALE	1		(+)	
	10/31-11/2/97	MALE	1		(+)	10/5/99 removed brain
	10/31-11/2/97	FEMALE	1		(+)	

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STRAIN	DOB	SEX	# OF MICE	CAGE ID	MOUSE # POSITIVE	COMMENTS			
(B6SJL)F1 Tg 2576	3/31/98	MALE	1	AA-24	(+)				
(B6SJL)F1 Tg 2576	6/9/98	MALE	1	AA-85	(+)				
(B6SJL)F1 Tg 2576	6/9/98	FEMALE	2	AA-80	(+)				
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-91	(+)				
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-92	(+)	9/12/98 wh, bk = Alz-KLH/CFA 10/24/98 same IFA 9/13/98 br = KLH control 10/24/98 same IFA			
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	2	AA-93	(+)	sick inner ear ??			
Tg2576 (B6SJL)F1	3/17-20/98	MALE	1	AA-18	(+)				
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-49	(+)	9/12/98 = Alz-KLH (was written on cage but not in cell) 10/24/98 same IFA			
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-50	(+)	9/13/98 = KLH control (this cell was not marked but cage was marked) 10/24/98 same IFA			
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-51	(+)	9/12/98 = Alz-KLH			
Tg2576 (B6SJL)F1	5/1/98	FEMALE	1	AA-53	(+)	(this cell had 9/13/98 = KLH control but cage was not marked; cage AA-50 was labeled instead)			
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/12/98 = Alz-KLH got same in IFA 10/24/98			
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/13/98 =KLH control 10/24/98 same IFA			
5A11 Exp/OLD Imm	5/14/97	FEMALE	3		(+)	DOA 9/16/97 2Bk= 5A11 1Br= Control then got Alz-KLH 9/28/98 got same in IFA 10/24/98			
5A11 Exp/OLD Imm	6/30/97	FEMALE	3		(+)	DOA 9/16/97 1Br=5A11 1Br= control 1Bk= control then one kill other Br got Alz-KLH 9/28/98 got same in IFA 10/24/98 and died a few min later anaphy? rough handling?			
	3/30/98	FEMALE	4	AA-22	3neg 1pos				
	3/17-20/98	FEMALE	2	AA-13	(+)				
	3/18/98	FEMALE	2	AA-20	(+)				
	5/14/97	MALE	1		(+)				
	8/13/97	FEMALE	4		2neg 2pos				
	6/30/97	MALE	1		(+)				
	10/31-11/2/97	MALE	1		(+)				
	10/31-11/2/97	FEMALE	1		(+)				
Tg2576 (B6SJL)F1	6/27/98	MALE	1	AA-99	(+)				

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Tg 2576 mice 10/24/98

STRAIN	DOB	SEX	# OF MICE	CAGE ID	MOUSE # POSITIVE	COMMENTS
(B6SJL)F1 Tg 2576	3/31/98	MALE	1	AA-24	(+)	
(B6SJL)F1 Tg 2576	6/9/98	MALE	1	AA-85	(+)	
(B6SJL)F1 Tg 2576	6/9/98	FEMALE	2	AA-80	(+)	
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-91	(+)	
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-92	(+)	9/12/98 wh, bk = Alz-KLH/CFA 10/24/98 same IFA 9/13/98 br = KLH control 10/24/98 same IFA
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	2	AA-93	(+)	sick inner ear ??
Tg2576 (B6SJL)F1	3/17-20/98	MALE	1	AA-18	(+)	
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-49	(+)	9/12/98 = Alz-KLH (was written on cage but not in cell) 10/24/98 same IFA
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-50	(+)	9/13/98 = KLH control (this cell was not marked but cage was marked) 10/24/98 same IFA
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-51	(+)	9/12/98 = Alz-KLH
Tg2576 (B6SJL)F1	5/1/98	FEMALE	1	AA-53	(+)	(this cell had 9/13/98 = KLH control but cage was not marked; cage AA-50 was labeled instead)
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/12/98 = Alz-KLH got same in IFA 10/24/98
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/13/98 =KLH control 10/24/98 same IFA
5A11 Exp/OLD Imm	5/14/97	FEMALE	3		(+)	DOA 9/16/97 2Bk= 5A11 1Br= Control then got Alz-KLH 9/28/98 got same in IFA 10/24/98
5A11 Exp/OLD Imm	6/30/97	FEMALE	3		(+)	DOA 9/16/97 1Br=5A11 1Br= control 1Bk= control then one kill other Br got Alz-KLH 9/28/98 got same in IFA 10/24/98 and died a few min later anaphy? rough handling?
	3/30/98	FEMALE	4	AA-22	3neg 1pos	
	3/17-20/98	FEMALE	2	AA-13	(+)	
	3/18/98	FEMALE	2	AA-20	(+)	
	5/14/97	MALE	1		(+)	
	8/13/97	FEMALE	4		2neg 2pos	
	6/30/97	MALE	1		(+)	

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Tg 2576 mice 9/28/98

STRAIN	DOB	SEX	# OF MICE	CAGE ID	MOUSE # POSITIVE	COMMENTS
(B6SJL)F1 Tg 2576	3/31/98	MALE	1	AA-24	(+)	
(B6SJL)F1 Tg 2576	6/9/98	MALE	1	AA-85	(+)	
(B6SJL)F1 Tg 2576	6/9/98	FEMALE	2	AA-80	(+)	
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-91	(+)	
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-92	(+)	9/12/98 wh, bk = Alz-KLH 9/13/98 br = KLH control
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	2	AA-93	(+)	sick inner ear
Tg2576 (B6SJL)F1	3/17-20/98	MALE	1	AA-18		
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-49		
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-50	(+)	
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-51	(+)	9/12/98 = Alz-KLH
Tg2576 (B6SJL)F1	5/1/98	FEMALE	1	AA-53	(+)	9/13/98 = KLH control
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/12/98 = Alz-KLH
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/13/98 = KLH control
5A11 Exp/OLD Imm	5/14/97	FEMALE	3		(+)	DOA 9/16/97 2Bk= 5A11 1Br= Control then got Alz-KLH 9/28/98
5A11 Exp/OLD Imm	6/30/97	FEMALE	3		(+)	DOA 9/16/97 Br=5A11 2Bk= control then one kill other got Alz-KLH 9/28/98

B001792

	3/30/98	FEMALE	4	AA-22	3neg 1pos	
	3/17-20/98	FEMALE	2	AA-13	(+)	
	3/18/98	FEMALE	2	AA-20	(+)	
	5/14/97	MALE	1		(+)	
	8/13/97	FEMALE	4		2neg 2pos	
	6/30/97	MALE	1		(+)	
	10/31-11/2/97	MALE	1		(+)	
	10/31-11/2/97	FEMALE	1		(+)	

STRAIN	BC #	ROOM #	DOB	SEX	# OF MICE	CAGE ID	MOUSE # POSITIVE	COMMENTS
B6(SJL)F1	JAX		10/31-11/2/98	MALE	1			removed from breeder Removed from Colony. Sac'd 6/23/99
B6(SJL)F1	JAX	069	5/3/99	FEMALE	1			
(B6SJL)F1 Tg 2576		068	6/15/98	MALE	1	AA-95	(+)	Moved to '068
(B6SJL)F1 Tg 2576		001	6/15/98	FEMALE	3	AA-91	(+)	Moved to room 001 on 6/99
(B6SJL)F1 Tg 2576		001	6/27/98	FEMALE	1	AA-97	(+)	Moved to room '001
(B6SJL)F1 Tg 2576	119	001	7/14/99	MALE	3	AD-11	all neg (-)	Moved to room '001
(B6SJL)F1 Tg 2576	119	001	7/14/99	FEMALE	3	AD-12	all neg (-)	Moved to room '001
(B6SJL)F1 Tg 2576		068	7/17-18/99	MALE	5	AD-13	#1, 4, (+)	#1 found dead 9/20/99
(B6SJL)F1 Tg 2576		068	7/17-18/99	MALE	5	AD-13	# 5 (+)	
(B6SJL)F1 Tg 2576		001	7/17-18/99	MALE	5	AD-13	#2, 3 (-)	Moved to room '001
(B6SJL)F1 Tg 2576		001	7/17-18/99	MALE	2	AD-14	all pos (+)	Moved to room '001
(B6SJL)F1 Tg 2576		001	7/17-18/99	FEMALE	5	AD-15	#4 pos (+)	Moved to room '001
(B6SJL)F1 Tg 2576		001	7/17-18/99	FEMALE	3	AD-16	all pos (+)	Moved to room '001
(B6SJL)F1 Tg 2576		001	7/17-18/99	FEMALE	3	AD-17	#1 pos (+)	Moved to room '001
(B6SJL)F1 Tg 2576	119	068	8/23/99	MALE	5			
(B6SJL)F1 Tg 2576	119	068	8/23/99	MALE	5			
(B6SJL)F1 Tg 2576	119	068	8/23/99	FEMALE	5			
(B6SJL)F1 Tg 2576	119	068	8/23/99	FEMALE	4			
(B6SJL)F1 Tg 2576	120	068	9/27/99	MALE	4			
(B6SJL)F1 Tg 2576	120	068	9/27/99	FEMALE	3			
(B6SJL)F1 Tg 2576	120	068	9/27/99	FEMALE	3			

B001794

	#	AD	DOB	#
	AA13	✓3/17/98		2
	AA24	✓8/23/98	3/31/98	1
	AA20	✓3/18/98		1
Treated	AA49	✓5/1/98		1
AZ KLH	AA50	✓5/1/98		1
Neg(-)	???	2/23/98	10/31/98 11/2/98	5
	AA85	6/9/98		1
	AA99	6/27/98		1
	??	12/22/97	8/13/97	2
	AA53	✓5/1/98		1
LZ-KLH ??	AA51	✓5/1/98		

B001797

3 ALZ

2 KLH

Tg 2576 mice 10/24/98

STRAIN	DOB	SEX	# OF MICE	CAGE ID	MOUSE # POSITIVE	COMMENTS
(B6SJL)F1 Tg 2576	3/31/98	MALE	1	AA-24	(+)	pos 7/27/99 *
(B6SJL)F1 Tg 2576	6/9/98	MALE	1	AA-85	(+)	
(B6SJL)F1 Tg 2576	6/9/98	FEMALE	2	AA-80	(+)	bled pos * 7/22/99 neg
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-91	(+)	Bled 7/22/99
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	3	AA-92	(+)	9/12/98 wh, bk = Alz-KLH/CFA 10/24/98 same IFA 9/13/98 br = KLH control 10/24/98 same IFA (1)
(B6SJL)F1 Tg 2576	6/15/98	FEMALE	2	AA-93	(+)	sick inner ear ??
Tg2576 (B6SJL)F1	3/17-20/98	MALE	1	AA-18	(+)	
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-49	(+)	9/12/98 = Alz-KLH (was written on cage but not in cell) 10/24/98 same IFA * Bled 7/22/99 pos
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-50	(+)	9/13/98 = KLH control (this cell was not marked but cage was marked) 10/24/98 same IFA Bled 7/26/99 neg (2)✓
Tg2576 (B6SJL)F1	5/1/98	MALE	1	AA-51	(+)	9/12/98 = Alz-KLH bled 7/22/99 neg ??
Tg2576 (B6SJL)F1	5/1/98	FEMALE	1 →	AA-53	(+)	(this cell had 9/13/98 = KLH control but cage was not marked; cage AA-50 was labeled instead)
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/12/98 = Alz-KLH got same in IFA 10/24/98 * Bled 7/22/99 positive
Tg2576 (B6SJL)F1	6/19/98	MALE	1	AA-87	(+)	9/13/98 =KLH control 10/24/98 same IFA ~~Bled~~ Bled 7/26/99 neg (3)✓
5A11 Exp/OLD Imm	old 5/14/97	FEMALE	3		(+)	DOA 9/16/97 2Bk= 5A11 1Br= Control then got Alz-KLH 9/28/98 got same in IFA 10/24/98
5A11 Exp/OLD Imm	old 6/30/97	FEMALE	3		(+)	DOA 9/16/97 1Br=5A11 1Br= control 1Bk= control then one kill other Br got Alz-KLH 9/28/98 got same in IFA 10/24/98 and died a few min later anaphy? rough handling?
	3/30/98	FEMALE	4	AA-22	3neg 1pos	
	3/17-20/98	FEMALE	2	AA-13	(+)	
	3/18/98	FEMALE	2	AA-20	(+)	
	5/14/97	MALE	1		(+)	
	8/13/97	FEMALE	4		2neg 2pos	7/22/99
	6/30/97	MALE	1		(+)	cant fen
	10/31-11/2/97	MALE	1		(+)	
	10/31-11/2/97	FEMALE	1		(+)	

B001798

SERI MORPHOLOGY

No. 001072

Service Requisition

DATE: 8/11/98
FUND #:
PRIN. INV.: Vic Raso
ORDERED BY: Vic Raso

Sample #: Transgenic Mouse Brain (+)

Blocks: 1

Species: Mouse

Fixative: paraform

Buffer: Phosp. X Cacodylate

INSTRUCTIONS

LM: Paraffin:

Routine:

Methacrylate:

ISH:

Immuno:

Frozen: X

TEM:

SEM:

Stains:

Slides Needed: 3-4

Special Instructions:

Date Needed:

Routine:

Grant:

Meeting

Paper:

Other:

MORPHOLOGY USE ONLY

Date Processed:

Date Completed and Delivered:

Date Billed:

B001801

SERI MORPHOLOGY No. 001090

Service Requisition

DATE: 9-8-98
FUND #: BBRI
PRIN. INV.: ~~B~~ Raso
ORDERED BY: Vic Raso

Sample #: Black Mouse 9-4-98 ♀ Tg T

Blocks: 1 Species:

Fixative: Buffer: Phosp. Cacodylate

INSTRUCTIONS

LM: Paraffin: Routine: Methacrylate:

ISH:

Immuno:

Frozen: TEM: SEM:

Stains:

Slides Needed:

Special Instructions:

Date Needed:

Routine: Grant: Meeting

Paper: Other:

MORPHOLOGY USE ONLY

Date Processed: Date Completed and Delivered: 9-9-98

Date Billed:

B001802

9/12/98

Immunize ALZ Tg mice

NH_2 - terminal - KLH - 1 mg/ml
Middle - peptide - KLH - 1 mg/ml
COOH - terminal - KLH - 1 mg/ml

use 20λ of each = 60λ = 60 μg / mouse
× 4 mice

immunize 4 mice ~~60λ + 400λ PBS + 460λ CFA~~

.2/mouse
0.2 ml

80λ each × 3 = 240λ + ~~200~~λ PBS + ~~440~~λ CFA = 880λ

use ~ 0.2 ml / mouse

9/28/98 - Immunize 2 Old Tg Mice

40λ each × 3 = 120λ + 120λ PB + 240λ CFA = 480λ

10/24/98 - Immunize 4 young + 2 old mice = 6

120λ of each antigen × 3 antigens = 360λ + 360λ PBS + 720λ IFA = 1.440 ml

use ~ 0.2 ml i.p. / mouse

10/24/98 - Boost KLH control mice (3)

240 μg/4 use 240λ KLH 1 mg/ml + 240λ PBS + 480λ IFA =

(over)

B001804

Ab = Brown ß ANIMAL FACILITY

SPECIES/STRAIN Tg 2576

SOURCE Colony ARRIVAL DATE 9·16·97

PROTOCOL # B0270800 ID # SB

DATE OF BIRTH 6/30/97 #/SEX 3♀

P.I. RASO FUND # BBRI

CONTACT NAME Christine EXT # 316

OTHER Tg + Br - ALZ-KLH 9/28/98

B001808

Tg2576 x B6(SJL)F1

6/19/98 #3 pos

♂ 1 9/12/98 all pos

CFA ALZ-KLH

Raso IFA ALZ-KLH - 10/24/98

IFA " " 7/27/99

" " " 9/18/99

AA-87

Tg2576 xB6(SJL)F1

6/19/98

#1 pos

1♂ CFA control KLH = 9/13/98 all pos

Raso IFA 10/24/98

IFA 7/27/99

FFA 9/18/99

AA-87

removed brains

10/23/99

THE SCHEP[redacted]EARCH INSTITUTE
ANIMAL FACILITY

SPECIES/STRAIN Tg 2756 x B6(SJL)F1

SOURCE ______ ARRIVAL DATE ______

PROTOCOL # ______ ID # ______

DATE OF BIRTH 5/1/98 #/SEX 1♂

P.I. RASO FUND # ______

CONTACT NAME ______ EXT # ______

OTHER AA-49 (+) * see back

CAGE CLINICAL R[redacted]

DATE	DIAGNOSIS	TREATMENT	INITIAL
9/12/98		CFA A12-KLH	
10/24/98		IFA A12-KLH	
7/27/99		IFA A12-KLH	
9/18/99		IFA A12-KLH	
3/30/00		IFA A12-KLH	

No serum

control KLH-Tg mouse

ALZ-KLH-Tg mouse 1

ALZ-KLH-Tg mouse 2

AT FAX 2100 18 SN 1112 01/21/99 11:57:38
ABSORBANCE MODE 12 PAGE 1 01/21/99 11:57:40
LOT NUMBER:________ EXP. DATE:________ USER:________
WAVELENGTHS=450NM 630NM

	1	2	3	4	5	6	7	8	9	10	11	12	
A	0.017	0.885	2.841	2.666	0.002	0.001	0.001	0.001	0.000	0.002	0.001	[illegible]	ALZ 1-43
B	0.014	0.847	2.951	2.640	0.001	0.001	0.002	0.001	0.001	[illegible]	[illegible]	0.001	ALZ 1-40
C	0.021	1.102	3.016	2.595	-0.001	0.000	0.000	-1.000	0.000	-0.000	[illegible]	0.000	ALZ 1-16
D	0.017	0.979	2.061	2.290	-0.000	-0.000	-0.000	-0.000	-0.000	-0.000	[illegible]	[illegible]	ALZ 14-2
E	0.017	0.864	3.171	2.951	-0.001	-0.000	-0.001	-0.001	0.001	0.000	-0.001	[illegible]	ALZ 34
F	-0.000	-0.001	-0.001	-0.000	-0.001	-0.000	-0.001	-0.001	-0.000	-0.000	-0.001	-0.001	

END OF RUN

No Serum

serum deluted 1/1,000

Control KLH-Tg mouse

ALZ-KLH Tg mouse 1

ALZ-KLH Tg mouse 2

ABSORBANCE MODE 11 PAGE 1 01/21/99 14:14:08
LOT NUMBER:________ EXP. DATE:________ USER:________
WAVELENGTHS=450NM 630NM

1	2	3	4	5	6	7	8	9	10	11	12	
0.017	0.127	1.618	0.678	0.001	0.001	0.002	0.002	0.001	0.002	0.001	0.000	ALZ 1-43
0.010	0.187	1.526	0.945	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.001	AB 1-40
0.014	0.232	1.461	0.673	-0.001	-0.000	-0.001	-0.001	-0.000	-0.001	-0.000	-0.000	Aβ 1-16
0.016	0.265	0.827	0.767	0.000	-0.001	0.000	-0.000	-0.001	-0.000	0.000	0.000	AB 14-25
0.014	0.247	2.145	0.897	0.012	0.011	0.012	0.009	0.017	0.013	0.014	0.014	AB 34-43
0.000	0.000	0.000	0.000	0.003	0.015	0.019	0.017	0.012	0.018	0.018	0.011	
[illegible]	[illegible]	[illegible]	0.000	0.006	0.010	0.011	0.008	0.010	0.007	0.012	[illegible]	
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	

get new control mouse

try something besides 0.5% BSA

use Ab@ 1/300

→ try 5% fish serum in first step

milk 10 ml ← 0.5 ml

B001814

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.018	0.50[illegible]	0.076	3.064	1.472	1.468	0.000	0.001	[illegible]	[illegible]	[illegible]	[illegible]
B	0.016	0.317	0.040	1.255	1.171	1.071	0.001	0.001	0.001	0.001	0.001	0.001
C	0.015	0.328	0.065	3.150	2.040	2.243	-0.002	0.000	-0.000	-0.001	0.000	-0.000
D	0.004	0.319	0.041	2.316	1.739	1.422	0.000	-0.001	0.000	0.000	-0.000	0.000
E	0.016	0.462	0.067	2.595	1.671	1.697	-0.000	-0.000	-0.001	-0.001	0.000	-0.001
F	-0.000	-0.000	-0.001	-0.000	-0.001	-0.001	-0.000	-0.001	-0.001	-0.001	-0.001	-0.000

END OF RUN

serum diluted 1/300 in 5% Fish Serum

anti-mouse γ 1/1000 in 5% Fish Serum

NorSerum

control KLH Tg mouse 1

control KLH Tg mouse 2

ALZKLH Tg mice

1 2 3

+ C+Y at either end

	1	2	3	4	5	6	7	8	9	10	11	12	
A	0.017	0.506	0.076	3.013	1.484	1.486	0.000	-0.001	-0.000	-0.000	[illegible]	[illegible]	Alz 1-16
B	0.014	0.314	0.038	1.258	1.180	1.072	-0.001	-0.001	-0.001	0.000	[illegible]	[illegible]	Alz 14-25
C	0.014	0.329	0.064	3.191	1.668	2.276	-0.002	0.000	0.000	0.000	[illegible]	[illegible]	ALZ 34-4
D	0.006	0.321	0.042	2.337	1.758	1.444	-0.000	-0.000	-0.000	-0.001	-0.001	0.000	AB 1-40
E	0.016	0.486	0.066	2.598	1.680	1.709	-0.000	0.001	0.000	0.000	-0.000	0.000	AB 1-43
F	-0.000	0.000	0.000	-0.000	-0.000	-0.000	0.000	0.000	0.000	0.000	0.000	0.000	

END OF RUN

in grant

503 items, 95.04 GB available

Name	Date Modified	Date Created	Size	Kind
Alz DNA 8/13/99 3	Fri, Aug 13, 1999, 10:47 AM	Fri, Aug 13, 1999, 10:47 AM	332 K	Multi-Analyst (256) document
Alz DNA 8/13/99 2	Fri, Aug 13, 1999, 10:47 AM	Fri, Aug 13, 1999, 10:47 AM	332 K	Multi-Analyst (256) document
Alz DNA 8/13/99 1	Fri, Aug 13, 1999, 10:27 AM	Fri, Aug 13, 1999, 10:27 AM	332 K	Multi-Analyst (256) document
Alz DNA 5	Thu, Aug 12, 1999, 12:28 PM	Thu, Aug 12, 1999, 12:28 PM	328 K	Multi-Analyst (256) document
Alz DNA 4	Thu, Aug 12, 1999, 12:27 PM	Thu, Aug 12, 1999, 12:27 PM	332 K	Multi-Analyst (256) document
Alz DNA 3	Thu, Aug 12, 1999, 11:47 AM	Thu, Aug 12, 1999, 11:47 AM	328 K	Multi-Analyst (256) document
Alz DNA 2	Thu, Aug 12, 1999, 11:43 AM	Thu, Aug 12, 1999, 11:43 AM	328 K	Multi-Analyst (256) document
Alz DNA 1	Thu, Aug 12, 1999, 11:04 AM	Thu, Aug 12, 1999, 11:04 AM	328 K	Multi-Analyst (256) document
KW-Y + pur KW6 fr as day2 NaOH	Fri, Jul 16, 1999, 9:25 AM	Fri, Jul 16, 1999, 9:25 AM	1.1 MB	Multi-Analyst (256) document
KW-Y + pure KW6 fr ascit day2 H.	Fri, Jul 16, 1999, 9:14 AM	Fri, Jul 16, 1999, 9:14 AM	1 MB	Multi-Analyst (256) document
KW-Y + pure KW6 from ascites	Thu, Jul 15, 1999, 9:06 AM	Thu, Jul 15, 1999, 9:06 AM	1.4 MB	Multi-Analyst (256) document
KW-Y + pure KW6 from ascites	Wed, Jul 14, 1999, 12:51 PM	Wed, Jul 14, 1999, 12:51 PM	1.8 MB	Multi-Analyst (256) document
KW-Y 6,tryp,K,pronase	Thu, Jul 1, 1999, 12:55 PM	Thu, Jul 1, 1999, 12:55 PM	1.1 MB	Multi-Analyst (256) document
KW-Y + enzymes	Thu, Jul 1, 1999, 8:41 AM	Thu, Jul 1, 1999, 8:41 AM	3 MB	Multi-Analyst (256) document
KW-Y +proteases	Thu, Jul 1, 1999, 8:29 AM	Thu, Jul 1, 1999, 8:29 AM	1.9 MB	Multi-Analyst (256) document
KW-Y new polyamid 6,T,M,P,C	Tue, Jun 22, 1999, 9:37 AM	Tue, Jun 22, 1999, 9:37 AM	1.5 MB	Multi-Analyst (256) document
KW-Y chymo KW6 Trypsin	Fri, Jun 18, 1999, 9:21 AM	Fri, Jun 18, 1999, 9:21 AM	648 K	Multi-Analyst (256) document
KW-Y pro pro chymo pro	Thu, Jun 17, 1999, 3:11 PM	Thu, Jun 17, 1999, 3:11 PM	808 K	Multi-Analyst (256) document
KW-Y pH 8 4 day	Thu, Jun 17, 1999, 1:05 PM	Thu, Jun 17, 1999, 1:05 PM	1 MB	Multi-Analyst (256) document
KW-Y ph 6 4day	Thu, Jun 17, 1999, 1:02 PM	Thu, Jun 17, 1999, 1:02 PM	1.5 MB	Multi-Analyst (256) document
KW-Y 6 and try mix co inc.	Thu, Jun 17, 1999, 12:18 PM	Thu, Jun 17, 1999, 12:18 PM	648 K	Multi-Analyst (256) document
KW-Y 6 tryp mix	Thu, Jun 17, 1999, 9:04 AM	Thu, Jun 17, 1999, 9:04 AM	264 K	Multi-Analyst (256) document
KW-Y block with KrW	Wed, Jun 16, 1999, 10:37 AM	Wed, Jun 16, 1999, 10:37 AM	1.6 MB	Multi-Analyst (256) document
KW-Y KrW block	Tue, Jun 15, 1999, 9:41 AM	Tue, Jun 15, 1999, 9:41 AM	904 K	Multi-Analyst (256) document
KW-Y pH 9	Tue, Jun 15, 1999, 8:30 AM	Tue, Jun 15, 1999, 8:30 AM	1.2 MB	Multi-Analyst (256) document
KW-Y pH 7.5	Tue, Jun 15, 1999, 8:27 AM	Tue, Jun 15, 1999, 8:27 AM	1.2 MB	Multi-Analyst (256) document
KW-Y pH 5.5	Tue, Jun 15, 1999, 8:09 AM	Tue, Jun 15, 1999, 8:09 AM	1.2 MB	Multi-Analyst (256) document
KW-Y 6, Sh, PBS. Tryp PO4, Na	Tue, Jun 15, 1999, 7:52 AM	Tue, Jun 15, 1999, 7:52 AM	2.6 MB	Multi-Analyst (256) document
KW-Y NaOH, HCl, pH 7 PO4	Sun, Jun 13, 1999, 12:04 PM	Sun, Jun 13, 1999, 12:04 PM	1.6 MB	Multi-Analyst (256) document
Raso,Letter	Tue, Jun 15, 1999, 1:18 PM	Sat, Jun 12, 1999, 1:42 PM	116 K	Microsoft Word 97-98 document
Alz 14, 17, 40-merHcl croplabel	Fri, Oct 23, 1998, 1:06 PM	Fri, Oct 23, 1998, 1:06 PM	120 K	Object-Image2.07 document
Alz 14, 17, 40-merHcllgexpcrop.	Fri, Oct 23, 1998, 12:57 PM	Fri, Oct 23, 1998, 12:57 PM	124 K	Object-Image2.07 document
Alz 14, 17, 40-merHcllgexpcrop	Fri, Oct 23, 1998, 12:48 PM	Fri, Oct 23, 1998, 12:48 PM	300 K	Multi-Analyst (256) document
Alz 14, 17, 40-mer Hcl 3day? le	Fri, Oct 23, 1998, 11:27 AM	Fri, Oct 23, 1998, 11:27 AM	1.3 MB	Multi-Analyst (256) document
Alz 14, 17, 40-mer Hcl long exp	Fri, Oct 23, 1998, 11:21 AM	Fri, Oct 23, 1998, 11:21 AM	1.3 MB	Multi-Analyst (256) document

503 items, 95.04 GB available

Name	Date Modified	Date Created	Size	Kind
Pbs 5A11 6E2 14mer 17mer 40mer	Mon, Oct 12, 1998, 11:08 AM	Mon, Oct 12, 1998, 11:08 AM	1.5 MB	Multi-Analyst (256) document
Alz 14-mer 17mer 1-40 HCl	Sat, Oct 10, 1998, 4:20 PM	Sat, Oct 10, 1998, 4:20 PM	784 K	Multi-Analyst (256) document
ALZ GRANT	Sat, Apr 26, 2003, 11:42 AM	Tue, Sep 15, 1998, 2:04 PM	—	folder
Catalytic Antibodies ?	Tue, Oct 20, 1998, 2:44 PM	Tue, Oct 20, 1998, 12:11 AM	1 MB	Microsoft Word 97-98 document
ALZ Catalytic SBIR unformated	Wed, Oct 14, 1998, 3:23 PM	Wed, Oct 14, 1998, 3:23 PM	1,004 K	Microsoft Word 1.x-5.x document
ALZ Vector Revision Grant	Wed, Oct 14, 1998, 1:12 PM	Thu, Oct 1, 1998, 5:08 PM	3.8 MB	Microsoft Word 1.x-5.x document
Vaccine to Modulate w low res p	Wed, Oct 14, 1998, 10:16 AM	Wed, Sep 30, 1998, 12:53 PM	1 MB	Microsoft Word 97-98 document
references	Fri, Sep 25, 1998, 4:35 PM	Fri, Sep 25, 1998, 4:26 PM	28 K	Microsoft Word 1.x-5.x document
End of Work Plan.doc	Fri, Sep 25, 1998, 12:57 PM	Fri, Sep 25, 1998, 12:57 PM	48 K	Microsoft Word 97-98 document
Plaques 2 copy 4	Thu, Sep 24, 1998, 5:07 PM	Thu, Sep 24, 1998, 5:07 PM	868 K	Photoshop® PICT file
plaque 1 copy 2	Thu, Sep 24, 1998, 5:05 PM	Thu, Sep 24, 1998, 5:05 PM	512 K	Photoshop® PICT file
ß Amyloid + antibody B & W EPS	Wed, Sep 16, 1998, 12:37 PM	Wed, Sep 16, 1998, 12:36 PM	72 K	Canvas™ 3.5.5 document
Vaccine to Modulate	Fri, Oct 9, 1998, 4:59 PM	Mon, Sep 14, 1998, 5:30 PM	1.5 MB	Microsoft Word 97-98 document
ffffff	Tue, Sep 15, 1998, 1:06 AM	Mon, Sep 14, 1998, 5:25 PM	92 K	Microsoft Word 97-98 document
ALZ abstract High Risk	Mon, Sep 14, 1998, 3:45 PM	Mon, Sep 14, 1998, 3:45 PM	12 K	Microsoft Word 1.x-5.x document
Cerebral Delivery	Wed, Oct 14, 1998, 2:10 PM	Mon, Sep 14, 1998, 3:12 PM	140 K	Microsoft Word 97-98 document
Catalytic Antibodies1	Mon, Oct 19, 1998, 3:51 PM	Mon, Sep 14, 1998, 3:11 PM	1 MB	Microsoft Word 97-98 document
Krs-fresh.doc	Wed, Sep 9, 1998, 10:54 AM	Wed, Sep 9, 1998, 10:54 AM	284 K	Microsoft Word 97-98 template
KRS-AF98.DOC	Tue, Sep 8, 1998, 10:49 AM	Tue, Sep 8, 1998, 4:20 PM	140 K	Microsoft Word 97-98 document
KRS-AF Kwik Guide.dot	Tue, Sep 8, 1998, 10:49 AM	Tue, Sep 8, 1998, 4:20 PM	236 K	Microsoft Word 97-98 template
Alz Vaccine High Risk	Wed, Sep 16, 1998, 2:52 PM	Sun, May 17, 1998, 3:03 PM	216 K	Microsoft Word 1.x-5.x document
ts/phosphonate/phosphoamid	Tue, Jun 3, 1997, 6:13 PM	Tue, Jun 3, 1997, 6:13 PM	12 K	ChemIntosh 3.4 document
Fig 8flip grey 3x.PICT	Fri, May 2, 1997, 2:07 PM	Mon, Dec 16, 1996, 3:48 PM	280 K	GraphicConverter PICT Picture
ts/phosphonate/phosphoamidate	Sun, Dec 8, 1996, 6:37 PM	Sun, Dec 8, 1996, 6:37 PM	12 K	ChemIntosh 3.4 document
TS phosphonate phosphoamidate	Sun, Dec 8, 1996, 6:33 PM	Sun, Dec 8, 1996, 6:33 PM	12 K	ChemIntosh 3.4 document
? KRS-AF98.DOC	Tue, Sep 8, 1998, 10:49 AM	Tue, Sep 8, 1998, 4:20 PM	140 K	Microsoft Word 97-98 document
KRS-AF Kwik Guide.dot	Tue, Sep 8, 1998, 10:49 AM	Tue, Sep 8, 1998, 4:20 PM	232 K	Microsoft Word 97-98 template
R17/5A11 WB (2) 24hr 5 min exp.	Tue, Jun 30, 1998, 3:19 PM	Tue, Jun 30, 1998, 3:19 PM	1.5 MB	Object-Image2.07 document
GPG & 17 mer	Mon, Apr 27, 1998, 11:24 AM	Mon, Apr 27, 1998, 11:24 AM	2.6 MB	Multi-Analyst (256) document
4/21/98	Tue, Apr 21, 1998, 2:09 PM	Tue, Apr 21, 1998, 2:09 PM	1.9 MB	Multi-Analyst (256) document
electrophoresis 5A11vs3H3 40vs1	Sun, Apr 12, 1998, 10:13 AM	Sun, Apr 12, 1998, 10:13 AM	1.2 MB	Multi-Analyst (256) document
electrophoresis	Sat, Apr 11, 1998, 2:11 PM	Sat, Apr 11, 1998, 2:11 PM	1.5 MB	Multi-Analyst (256) document
TLC on reg. cell, & DEAE cell.	Thu, Apr 9, 1998, 3:54 PM	Thu, Apr 9, 1998, 3:54 PM	1.7 MB	Multi-Analyst (256) document
GPG TLC w/ inhibitor 4/9/98	Thu, Apr 9, 1998, 1:45 PM	Thu, Apr 9, 1998, 1:45 PM	1.3 MB	Multi-Analyst (256) document
GPG TLC 4/9/98	Thu, Apr 9, 1998, 12:10 PM	Thu, Apr 9, 1998, 12:10 PM	1.5 MB	Multi-Analyst (256) document

503 items, 95.04 Gb available

Name	Date Modified	Date Created	Size	Kind
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GPG V3 TLC	Wed, Apr 8, 1998, 2:48 PM	Wed, Apr 8, 1998, 2:48 PM	1.3 MB	Multi-Analyst (256) document
GPG + 7D3 +7D3+Ovoinhibitor	Thu, Mar 26, 1998, 4:04 PM	Thu, Mar 26, 1998, 4:04 PM	584 K	Multi-Analyst (256) document
GPG + PBS,7D3,7D3+4A proinhib	Mon, Mar 23, 1998, 11:14 AM	Mon, Mar 23, 1998, 11:14 AM	776 K	Multi-Analyst (256) document
A1-40 tween ipnegTg 11d 20mex	Sat, Mar 21, 1998, 1:04 PM	Sat, Mar 21, 1998, 1:04 PM	1.5 MB	Multi-Analyst (256) document
GPG,V3,A1-40 +7D3 +/- 4A inhibi	Sat, Mar 21, 1998, 11:41 AM	Sat, Mar 21, 1998, 11:41 AM	2 MB	Multi-Analyst (256) document
GPG + 7D3 + prot inhib 18h	Fri, Mar 20, 1998, 11:51 AM	Fri, Mar 20, 1998, 11:51 AM	1.5 MB	Multi-Analyst (256) document
GPG + 7D3 + prot inhib 1h incub	Thu, Mar 19, 1998, 4:23 PM	Thu, Mar 19, 1998, 4:23 PM	776 K	Multi-Analyst (256) document
GPG +7D3 + protease inhibitors	Thu, Mar 19, 1998, 11:32 AM	Thu, Mar 19, 1998, 11:32 AM	1.7 MB	Multi-Analyst (256) document
GPG 0.5N NaOH PBS,7D3,3H3	Wed, Mar 18, 1998, 6:03 PM	Wed, Mar 18, 1998, 6:03 PM	680 K	Multi-Analyst (256) document
GPG 1M AA	Wed, Mar 18, 1998, 5:30 PM	Wed, Mar 18, 1998, 5:30 PM	904 K	Multi-Analyst (256) document
GPG & V3 2N & .1N NaOH + 3H3	Wed, Mar 18, 1998, 4:29 PM	Wed, Mar 18, 1998, 4:29 PM	1.3 MB	Multi-Analyst (256) document
GPG & V3 in iM Acetic Acid	Wed, Mar 18, 1998, 3:58 PM	Wed, Mar 18, 1998, 3:58 PM	1.5 MB	Multi-Analyst (256) document
A14-25CY PBS,7D3,5A11a,5A11b	Wed, Mar 18, 1998, 1:46 PM	Wed, Mar 18, 1998, 1:46 PM	1.1 MB	Multi-Analyst (256) document
V3 & GPG PBS,7D3,5A11,3H3	Wed, Mar 18, 1998, 11:39 AM	Wed, Mar 18, 1998, 11:39 AM	2.6 MB	Multi-Analyst (256) document
A14-25CY & V3 TLC ~24h 20m exp	Tue, Mar 17, 1998, 11:23 AM	Tue, Mar 17, 1998, 11:23 AM	1.6 MB	Multi-Analyst (256) document
A14-25CY and V3 loop TLC	Tue, Mar 17, 1998, 10:04 AM	Tue, Mar 17, 1998, 10:04 AM	3.2 MB	Multi-Analyst (256) document
A14-25 P,7D3,5A11a&b NH3,pH7 1h	Mon, Mar 16, 1998, 12:28 PM	Mon, Mar 16, 1998, 12:28 PM	1.3 MB	Multi-Analyst (256) document
A1-40 tween ipposTg 48h 20m exp	Sat, Mar 14, 1998, 11:11 AM	Sat, Mar 14, 1998, 11:11 AM	1.5 MB	Multi-Analyst (256) document
A1-40 tween ipposTg 24h 20m exp	Fri, Mar 13, 1998, 11:18 AM	Fri, Mar 13, 1998, 11:18 AM	1.5 MB	Multi-Analyst (256) document
A1-40 tween ipposTg 6.5h 25m ex	Thu, Mar 12, 1998, 5:51 PM	Thu, Mar 12, 1998, 5:51 PM	1.5 MB	Multi-Analyst (256) document
A1-40 tween ipposTG 1h 20m exp	Thu, Mar 12, 1998, 12:18 PM	Thu, Mar 12, 1998, 12:18 PM	1.5 MB	Multi-Analyst (256) document
A1-40 tween ipnegTg 48h 20m ex	Thu, Mar 12, 1998, 11:37 AM	Thu, Mar 12, 1998, 11:37 AM	1.5 MB	Multi-Analyst (256) document
A1-40 tween ipnegTg 24h 20m exp	Wed, Mar 11, 1998, 11:42 AM	Wed, Mar 11, 1998, 11:42 AM	1.5 MB	Multi-Analyst (256) document
A1-40 tween ipnegTg 6.5h 20m ex	Tue, Mar 10, 1998, 6:17 PM	Tue, Mar 10, 1998, 6:17 PM	1.7 MB	Multi-Analyst (256) document
A1-40 tween ipnegTg 6h 20min	Tue, Mar 10, 1998, 5:37 PM	Tue, Mar 10, 1998, 5:37 PM	1.5 MB	Multi-Analyst (256) document
A1-40 tween ipnegTg 1h 20 m	Tue, Mar 10, 1998, 12:45 PM	Tue, Mar 10, 1998, 12:45 PM	1.5 MB	Multi-Analyst (256) document
R17/5A11 ip posTg 48h 20min	Fri, Mar 6, 1998, 11:31 AM	Fri, Mar 6, 1998, 11:31 AM	1.5 MB	Multi-Analyst (256) document
R17/5A11 ip posTg 24h 20min	Thu, Mar 5, 1998, 11:45 AM	Thu, Mar 5, 1998, 11:45 AM	1.5 MB	Multi-Analyst (256) document
R17/5A11 ip posTg 6h 20min	Wed, Mar 4, 1998, 5:53 PM	Wed, Mar 4, 1998, 5:53 PM	1.5 MB	Multi-Analyst (256) document
GPG digest 48h + enz. in HCl	Wed, Mar 4, 1998, 1:01 PM	Wed, Mar 4, 1998, 1:01 PM	1.1 MB	Multi-Analyst (256) document
GPG digest O/N + enz. ph7buffer	Tue, Mar 3, 1998, 4:42 PM	Tue, Mar 3, 1998, 4:42 PM	1.7 MB	Multi-Analyst (256) document
GPG digest O/N + enzymes	Tue, Mar 3, 1998, 3:28 PM	Tue, Mar 3, 1998, 3:28 PM	2 MB	Multi-Analyst (256) document
GPG digest O/N ph7 buffer 20min	Tue, Mar 3, 1998, 12:11 PM	Tue, Mar 3, 1998, 12:11 PM	1.5 MB	Multi-Analyst (256) document
GPG digest <1hr O/N exp	Tue, Mar 3, 1998, 10:51 AM	Tue, Mar 3, 1998, 10:51 AM	2 MB	Multi-Analyst (256) document

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Phosphorimager Alz Files

503 items, 95.04 GB available

Name	Date Modified	Date Created	Size	Kind
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GPG digest O/N 20min exp	Tue, Mar 3, 1998, 10:06 AM	Tue, Mar 3, 1998, 10:06 AM	2 MB	Multi-Analyst (256) document
GPG digest <1hr 2min exp	Mon, Mar 2, 1998, 6:08 PM	Mon, Mar 2, 1998, 6:08 PM	1.3 MB	Multi-Analyst (256) document
NacetylGPGRAFY pH7, NH4OH, HCl	Mon, Mar 2, 1998, 4:09 PM	Mon, Mar 2, 1998, 4:09 PM	1.6 MB	Multi-Analyst (256) document
R17/5A11 ip negTg 120h 20min	Mon, Mar 2, 1998, 11:25 AM	Mon, Mar 2, 1998, 11:25 AM	1.5 MB	Multi-Analyst (256) document
R17/5A11 ip negTg 48h 20min	Fri, Feb 27, 1998, 12:43 PM	Fri, Feb 27, 1998, 12:43 PM	1.5 MB	Multi-Analyst (256) document
R17/5A11 ip negTg 24h 20min	Thu, Feb 26, 1998, 12:42 PM	Thu, Feb 26, 1998, 12:42 PM	1.5 MB	Multi-Analyst (256) document
R17/5A11 ip negTg 6hr 20min exp	Wed, Feb 25, 1998, 6:40 PM	Wed, Feb 25, 1998, 6:40 PM	1.5 MB	Multi-Analyst (256) document
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A14-25CY pases NH4OH pH7 PO4	Mon, Feb 23, 1998, 11:23 AM	Mon, Feb 23, 1998, 11:23 AM	2.6 MB	Multi-Analyst (256) document
A14-25CY Pases NH4OH pH7PO4	Sun, Feb 22, 1998, 5:48 PM	Sun, Feb 22, 1998, 5:48 PM	2.2 MB	Multi-Analyst (256) document
A14-25CY +PBS5A117D3A?	Sun, Feb 22, 1998, 3:31 PM	Sun, Feb 22, 1998, 3:31 PM	1.1 MB	Multi-Analyst (256) document
A1-40+ Pases +Chymo NH4OH	Fri, Feb 20, 1998, 3:44 PM	Fri, Feb 20, 1998, 3:44 PM	1.1 MB	Multi-Analyst (256) document
A1-40+Pases +TpckTry +chymo HCl	Fri, Feb 20, 1998, 10:31 AM	Fri, Feb 20, 1998, 10:31 AM	972 K	Multi-Analyst (256) document
A1-40 TPCK Try Chymo HCl	Thu, Feb 19, 1998, 6:39 PM	Thu, Feb 19, 1998, 6:39 PM	780 K	Multi-Analyst (256) document
Ratfeb18/data	Thu, Feb 19, 1998, 1:35 PM	Thu, Feb 19, 1998, 1:35 PM	8 K	document
A1-40 Pases NH4oh vs HCl	Thu, Feb 19, 1998, 10:23 AM	Thu, Feb 19, 1998, 10:23 AM	1.7 MB	Multi-Analyst (256) document
A1-40 Pases, NH4OH vs HCl	Wed, Feb 18, 1998, 2:52 PM	Wed, Feb 18, 1998, 2:52 PM	1.5 MB	Multi-Analyst (256) document
A1-40+PasesPbsElProtPronTry5A11	Wed, Feb 18, 1998, 1:34 PM	Wed, Feb 18, 1998, 1:34 PM	780 K	Multi-Analyst (256) document
A1-40 PBS5A117D3 dil NH4OH	Tue, Feb 17, 1998, 3:49 PM	Tue, Feb 17, 1998, 3:49 PM	776 K	Multi-Analyst (256) document
A1-40 PBS5A117D3 conc NH4OH	Tue, Feb 17, 1998, 2:05 PM	Tue, Feb 17, 1998, 2:05 PM	680 K	Multi-Analyst (256) document
A1-40 PBS5A117D3 NaOH 20min exp	Tue, Feb 17, 1998, 12:27 PM	Tue, Feb 17, 1998, 12:27 PM	584 K	Multi-Analyst (256) document
A1-40 PBS5A117D3	Tue, Feb 17, 1998, 11:28 AM	Tue, Feb 17, 1998, 11:28 AM	2.8 MB	Multi-Analyst (256) document
A1-40 pbs 5A11 7D3 HCl Borate	Mon, Feb 16, 1998, 2:07 PM	Mon, Feb 16, 1998, 2:07 PM	812 K	Multi-Analyst (256) document
Alz1-40 +5A11 or 7D3 PO4 or MeH	Mon, Feb 16, 1998, 12:43 PM	Mon, Feb 16, 1998, 12:43 PM	1.1 MB	Multi-Analyst (256) document
A1-40 on polyamide 50% MeH2O	Sun, Feb 15, 1998, 6:42 PM	Sun, Feb 15, 1998, 6:42 PM	332 K	Multi-Analyst (256) document
RETARDING GEL 18 HR EXP	Sat, Feb 14, 1998, 11:17 AM	Sat, Feb 14, 1998, 11:17 AM	1.1 MB	Multi-Analyst (256) document
RETARDING GEL 30MIN EXP	Fri, Feb 13, 1998, 6:13 PM	Fri, Feb 13, 1998, 6:13 PM	776 K	Multi-Analyst (256) document
R17/5A11 WB 12 days vent 20 min	Wed, Feb 4, 1998, 1:12 PM	Wed, Feb 4, 1998, 1:12 PM	3.7 MB	Multi-Analyst (256) document
ALZ 1-40 tween 48hr. 20 min exp	Wed, Feb 4, 1998, 11:13 AM	Wed, Feb 4, 1998, 11:13 AM	1.5 MB	Multi-Analyst (256) document
Alz 1-40 tween 24hr. 20min exp	Tue, Feb 3, 1998, 11:38 AM	Tue, Feb 3, 1998, 11:38 AM	1.5 MB	Multi-Analyst (256) document
saved by Toli 02/02	Mon, Feb 2, 1998, 8:01 PM	Mon, Feb 2, 1998, 8:01 PM	2.5 MB	Multi-Analyst (256) document
Alz 1-40 tween 6hr. 20 min exp	Mon, Feb 2, 1998, 5:41 PM	Mon, Feb 2, 1998, 5:41 PM	1.5 MB	Multi-Analyst (256) document
Alz 1-40 tween 1hr. 20min exp	Mon, Feb 2, 1998, 12:35 PM	Mon, Feb 2, 1998, 12:35 PM	1.5 MB	Multi-Analyst (256) document
R17/5A11 7D3/1G7 gel 18hr exp	Thu, Jan 29, 1998, 10:25 AM	Thu, Jan 29, 1998, 10:25 AM	780 K	Multi-Analyst (256) document

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Name	Date Modified	Date Created	Size	Kind
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R17/5A11 (3) 96hr 20 min exp	Wed, Jan 28, 1998, 12:05 PM	Wed, Jan 28, 1998, 12:05 PM	1.5 MB	Multi-Analyst (256) document
standards(3) with regression	Tue, Jan 27, 1998, 5:16 PM	Tue, Jan 27, 1998, 5:16 PM	584 K	Multi-Analyst (256) document
standards(3)	Tue, Jan 27, 1998, 3:59 PM	Tue, Jan 27, 1998, 3:59 PM	584 K	Multi-Analyst (256) document
standards(2) with regression	Tue, Jan 27, 1998, 3:05 PM	Tue, Jan 27, 1998, 3:05 PM	1.1 MB	Multi-Analyst (256) document
standard curve 1/27/98	Tue, Jan 27, 1998, 2:49 PM	Tue, Jan 27, 1998, 2:49 PM	8 K	document
standards(2)	Tue, Jan 27, 1998, 12:21 PM	Tue, Jan 27, 1998, 12:21 PM	1.1 MB	Multi-Analyst (256) document
standards	Tue, Jan 27, 1998, 11:27 AM	Tue, Jan 27, 1998, 11:27 AM	648 K	Multi-Analyst (256) document
R17/5A11 (3) 72hr 20 min exp	Mon, Jan 26, 1998, 12:08 PM	Mon, Jan 26, 1998, 12:08 PM	1.5 MB	Multi-Analyst (256) document
R17/5A11 (3) 48hr 20 min exp M	Sun, Jan 25, 1998, 11:45 AM	Sun, Jan 25, 1998, 11:45 AM	1.5 MB	Multi-Analyst (256) document
R17/5A11 (3) 24hr 20 min exp	Sat, Jan 24, 1998, 11:51 AM	Sat, Jan 24, 1998, 11:51 AM	1.5 MB	Multi-Analyst (256) document
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screen 2(a)	Fri, Jan 23, 1998, 3:47 PM	Fri, Jan 23, 1998, 3:47 PM	520 K	Multi-Analyst (256) document
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screen 1	Fri, Jan 23, 1998, 1:49 PM	Fri, Jan 23, 1998, 1:48 PM	10 MB	Multi-Analyst (256) document
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R17/5A11 WB (2) 96hr 5 min exp	Tue, Jan 20, 1998, 1:20 PM	Tue, Jan 20, 1998, 1:20 PM	3.2 MB	Multi-Analyst (256) document
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R17/5A11 (2) 1hr 20 min exp	Fri, Jan 16, 1998, 1:28 PM	Fri, Jan 16, 1998, 1:28 PM	1.5 MB	Multi-Analyst (256) document
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ALZ1-40 24hr 20+min exp	Thu, Jan 15, 1998, 11:16 AM	Thu, Jan 15, 1998, 11:16 AM	1.5 MB	Multi-Analyst (256) document
ALZ1-40 6hr 20 min exp	Wed, Jan 14, 1998, 5:35 PM	Wed, Jan 14, 1998, 5:35 PM	1.5 MB	Multi-Analyst (256) document
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R17/5A11 72hr 20 min exp	Tue, Jan 13, 1998, 11:31 AM	Tue, Jan 13, 1998, 11:31 AM	1.5 MB	Multi-Analyst (256) document
Data	Mon, Jan 12, 1998, 11:33 AM	Mon, Jan 12, 1998, 11:33 AM	8 K	document
R17/5A11 48hr 20 min exp	Mon, Jan 12, 1998, 10:55 AM	Mon, Jan 12, 1998, 10:55 AM	1.5 MB	Multi-Analyst (256) document
R17/5A11 24hr 5 min exp	Sun, Jan 11, 1998, 11:07 AM	Sun, Jan 11, 1998, 11:07 AM	1.5 MB	Multi-Analyst (256) document
R17/5A11 24hr 20 min exp	Sun, Jan 11, 1998, 11:00 AM	Sun, Jan 11, 1998, 11:00 AM	1.5 MB	Multi-Analyst (256) document
R17/5A11 6hr 20 min exp	Sat, Jan 10, 1998, 4:53 PM	Sat, Jan 10, 1998, 4:53 PM	1.5 MB	Multi-Analyst (256) document
R17/5A11 1hr 20 min exp	Sat, Jan 10, 1998, 11:52 AM	Sat, Jan 10, 1998, 11:52 AM	1.5 MB	Multi-Analyst (256) document

503 items, 95.[illegible]4 GB available

Name	Date Modified	Date Created	Size	Kind
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mouse 6 hr 20 min exp	Mon, Dec 22, 1997, 12:35 PM	Mon, Dec 22, 1997, 12:35 PM	1.5 MB	Multi-Analyst (256) document
mouse 1 hr 20 min exp	Mon, Dec 22, 1997, 12:35 PM	Mon, Dec 22, 1997, 12:35 PM	1.3 MB	Multi-Analyst (256) document
7D3/1G1 24hr 20 min exp	Mon, Dec 22, 1997, 12:27 PM	Mon, Dec 22, 1997, 12:27 PM	1.5 MB	Multi-Analyst (256) document
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7D3/1G1 1hr 20min exp	Mon, Dec 22, 1997, 12:25 PM	Mon, Dec 22, 1997, 12:25 PM	1.5 MB	Multi-Analyst (256) document
M/24hr	Fri, Dec 19, 1997, 2:51 PM	Fri, Dec 19, 1997, 2:51 PM	584 K	Object-Image2.07 document
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M/1hr	Fri, Dec 19, 1997, 2:49 PM	Fri, Dec 19, 1997, 2:49 PM	488 K	Object-Image2.07 document
R17/24hr	Fri, Dec 19, 1997, 2:46 PM	Fri, Dec 19, 1997, 2:46 PM	532 K	Object-Image2.07 document
R1	Fri, Dec 19, 1997, 12:37 PM	Fri, Dec 19, 1997, 12:37 PM	3.1 MB	Object-Image2.07 document
M1	Fri, Dec 19, 1997, 12:32 PM	Fri, Dec 19, 1997, 12:32 PM	824 K	Object-Image2.07 document
ALZ Vector	Wed, Jan 21, 1998, 4:00 PM	Tue, Nov 25, 1997, 5:13 PM	1.9 MB	Microsoft Word 1.x-5.x document

59 items, 95.04 GB available

Name	Date Modified	Date Created	Size	Kind
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plaque 1	Tue, Aug 18, 1998, 11:43 AM	Tue, Aug 18, 1998, 11:43 AM	3.6 MB	Photoshop® TIFF file
R17/5A11 WB (2) 24hr 5 col.tiff	Tue, Jun 30, 1998, 3:25 PM	Tue, Jun 30, 1998, 3:25 PM	1.5 MB	Object-Image2.07 document
R17/5A11 WB (2) 24hr 5 min exp.	Tue, Jun 30, 1998, 3:19 PM	Tue, Jun 30, 1998, 3:19 PM	1.5 MB	Object-Image2.07 document
A1-40 tween ipnegTg 48h 20m ex	Thu, Mar 12, 1998, 11:37 AM	Thu, Mar 12, 1998, 11:37 AM	1.5 MB	Multi-Analyst (256) document
Alz21-40	Mon, Feb 16, 1998, 9:49 AM	Mon, Feb 16, 1998, 9:49 AM	8 K	EditSeq document
Alz1-20	Mon, Feb 16, 1998, 9:49 AM	Mon, Feb 16, 1998, 9:49 AM	8 K	EditSeq document
Vectorized anti-Aß EPSF.PICT	Sat, Feb 7, 1998, 4:17 PM	Sat, Feb 7, 1998, 4:17 PM	36 K	GraphicConverter PICT Picture
Vector anti-Aß Imaging8bit.tif	Sat, Feb 7, 1998, 1:32 PM	Sat, Feb 7, 1998, 1:32 PM	140 K	Canvas™ 3.5.5 document
Vectorized anti-Aß Imaging.tif	Sat, Feb 7, 1998, 9:01 AM	Sat, Feb 7, 1998, 9:01 AM	408 K	Canvas™ 3.5.5 document
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compos hrs + 5m ex 24h IP.TIFF	Sun, Jan 18, 1998, 11:07 AM	Sun, Jan 18, 1998, 11:07 AM	700 K	GraphicConverter TIFF Picture
composite w hrs + 5min exp 24h	Sun, Jan 18, 1998, 10:06 AM	Sun, Jan 18, 1998, 10:06 AM	684 K	Object-Image2.07 document
R17/5A11 24hr 5 min exp.tiff	Sun, Jan 18, 1998, 10:02 AM	Sun, Jan 18, 1998, 10:02 AM	644 K	Object-Image2.07 document
composite w hrs	Sat, Jan 17, 1998, 2:48 PM	Sat, Jan 17, 1998, 2:48 PM	684 K	Object-Image2.07 document
composite 1	Sat, Jan 17, 1998, 1:34 PM	Sat, Jan 17, 1998, 1:34 PM	696 K	Object-Image2.07 document
untitled 3	Sat, Jan 17, 1998, 1:28 PM	Sat, Jan 17, 1998, 1:28 PM	4.9 MB	Multi-Analyst (256) document
7D3/1G1 1hr 20min exp.tiff	Sat, Jan 17, 1998, 1:05 PM	Sat, Jan 17, 1998, 1:04 PM	632 K	Object-Image2.07 document
7D3/1G1 6hr 20 min exp.tiff	Sat, Jan 17, 1998, 1:04 PM	Sat, Jan 17, 1998, 1:04 PM	632 K	Object-Image2.07 document
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R17/5A11 48hr 20 min exp.tiff	Sat, Jan 17, 1998, 1:00 PM	Sat, Jan 17, 1998, 1:00 PM	644 K	Object-Image2.07 document
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fitc + label.tif	Tue, Dec 30, 1997, 9:57 AM	Tue, Dec 30, 1997, 9:52 AM	792 K	Photoshop® TIFF file
R17/5D5 + NS1 cells.tif	Tue, Dec 30, 1997, 9:11 AM	Tue, Dec 30, 1997, 9:11 AM	20 K	Photoshop® TIFF file
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R17/24hr	Fri, Dec 19, 1997, 2:46 PM	Fri, Dec 19, 1997, 2:46 PM	532 K	Object-Image2.07 document
R17/7hr	Fri, Dec 19, 1997, 2:45 PM	Fri, Dec 19, 1997, 2:45 PM	456 K	Object-Image2.07 document
R17/1hr	Fri, Dec 19, 1997, 2:44 PM	Fri, Dec 19, 1997, 2:44 PM	508 K	Object-Image2.07 document
untitled 2.PICT	Fri, Dec 19, 1997, 1:58 PM	Fri, Dec 19, 1997, 1:57 PM	164 K	GraphicConverter PICT Picture
R24	Fri, Dec 19, 1997, 12:38 PM	Fri, Dec 19, 1997, 12:38 PM	3.2 MB	Object-Image2.07 document

B001940

59 items, 95.04 GB available

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M1	Fri, Dec 19, 1997, 12:32 PM	Fri, Dec 19, 1997, 12:32 PM	824 K	Object-Image2.07 document
m6	Fri, Dec 19, 1997, 12:30 PM	Fri, Dec 19, 1997, 12:30 PM	876 K	Object-Image2.07 document
untitled.PICT	Fri, Dec 19, 1997, 2:00 PM	Fri, Dec 19, 1997, 12:00 PM	360 K	GraphicConverter PICT Picture
M R17block 24 hrs. 20 min. exp.	Fri, Dec 19, 1997, 11:10 AM	Fri, Dec 19, 1997, 11:10 AM	1.3 MB	Object-Image2.07 document
M R17 block 7hr 20 min exp.tiff	Fri, Dec 19, 1997, 11:10 AM	Fri, Dec 19, 1997, 11:10 AM	480 K	Object-Image2.07 document
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mouse 6 hr 20 min exp.tiff	Fri, Dec 19, 1997, 11:09 AM	Fri, Dec 19, 1997, 11:09 AM	704 K	Object-Image2.07 document
mouse 1 hr 20 min exp.tiff	Fri, Dec 19, 1997, 11:09 AM	Fri, Dec 19, 1997, 11:09 AM	640 K	Object-Image2.07 document
M R17 block 7hr 20 min 2.tiff	Fri, Dec 19, 1997, 9:08 AM	Fri, Dec 19, 1997, 9:08 AM	480 K	Object-Image2.07 document
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ß-Amyloid 1-43	Sat, Dec 13, 1997, 9:31 AM	Sat, Dec 13, 1997, 9:31 AM	8 K	EditSeq document
Capillary Depletion Pict	Wed, Dec 10, 1997, 3:20 PM	Wed, Dec 10, 1997, 3:20 PM	8 K	document
Capillary Depletion 2	Wed, Dec 10, 1997, 3:20 PM	Wed, Dec 10, 1997, 3:20 PM	56 K	KaleidaGraph document
cap. depletion data	Wed, Dec 10, 1997, 3:10 PM	Mon, Dec 8, 1997, 3:18 PM	12 K	KaleidaGraph document
Alz APP AA seq part?	Sat, Jun 21, 1997, 12:45 PM	Sat, Jun 21, 1997, 12:45 PM	8 K	EditSeq document
Alz APP	Sat, Jun 21, 1997, 12:38 PM	Sat, Jun 21, 1997, 12:38 PM	8 K	SimpleText text document
Alz APP seq part?	Sat, Jun 21, 1997, 12:29 PM	Sat, Jun 21, 1997, 12:29 PM	8 K	EditSeq document
alz precipitate	Mon, Jun 9, 1997, 10:48 AM	Sun, Jun 8, 1997, 2:32 PM	1.7 MB	GraphicConverter TIFF Picture
ALZ phenylalanine statine.PIC	Wed, Jun 4, 1997, 9:38 AM	Wed, Jun 4, 1997, 9:38 AM	32 K	ChemIntosh 3.4 document
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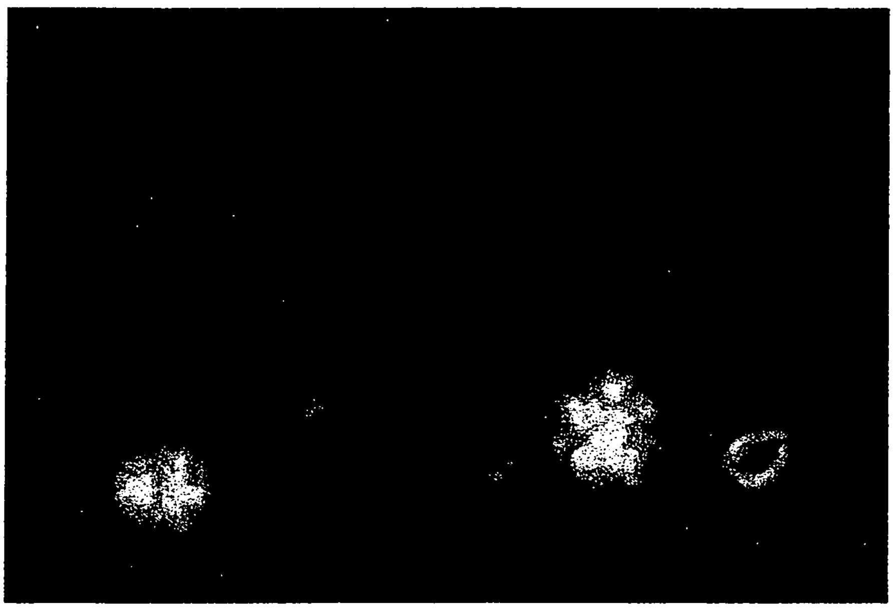
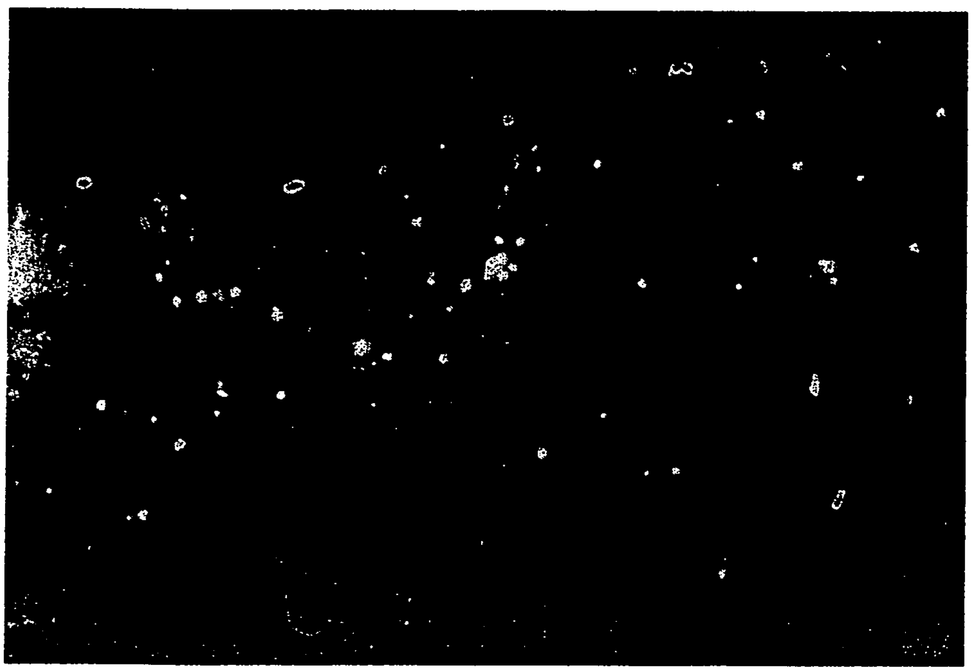
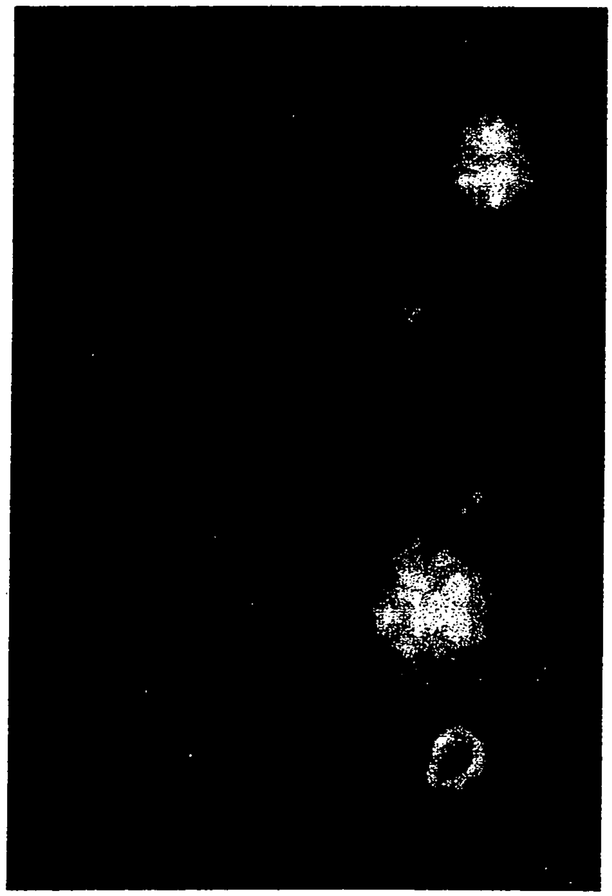


9/10/97

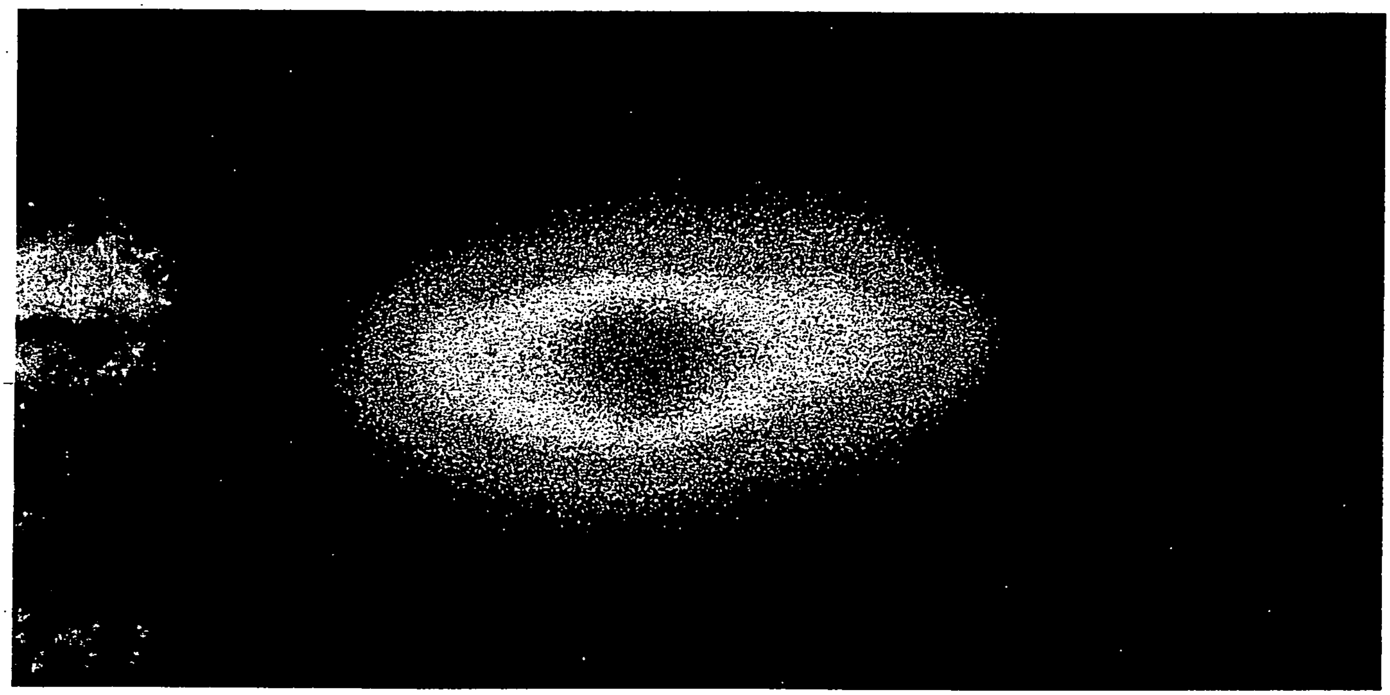


10/8/98

B001943



10/13/98



6/30/07

6/30/08

Created: Monday, February 16, 1998 9:45 AM

10 20 30 40

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2/16/98

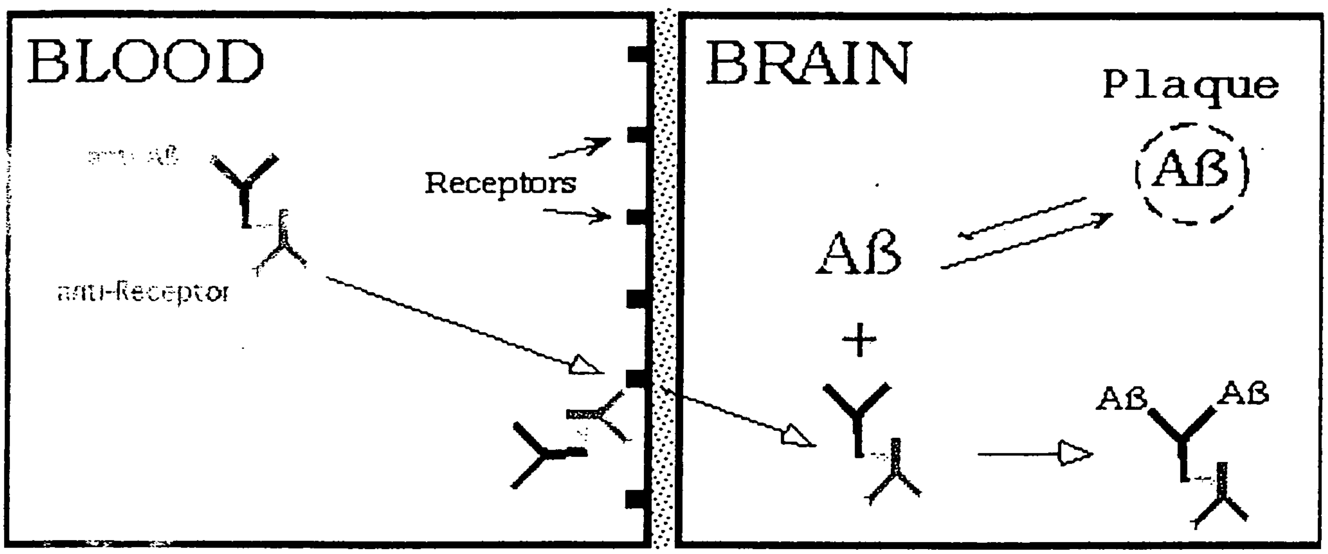
Created: Monday, February 16, 1998 9:46 AM

10 20 30 40

DAEFRHDSGYEVHHQKLVF 19

2/16/98

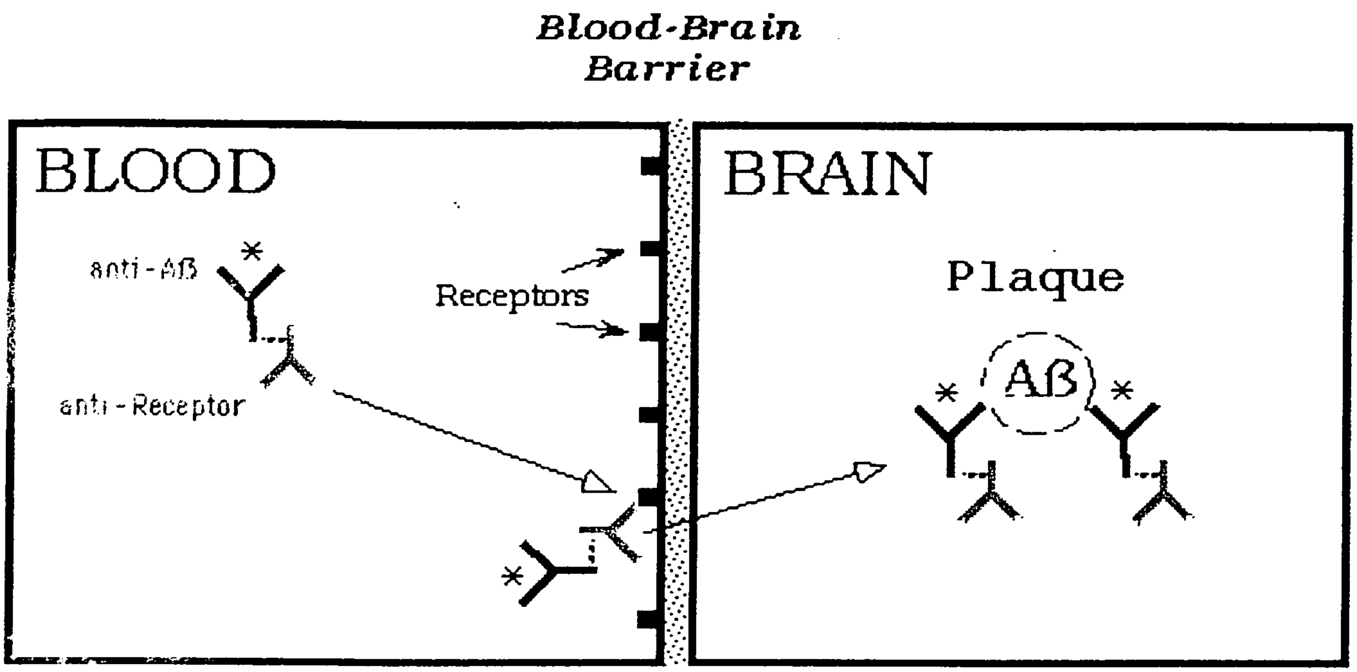
Blood-Brain Barrier



1 Transcytosis Across the Brain Capillary Endothelial

2 Dissolve Plaques by Depletion of Soluble Aß Level in Brain

2/7/98

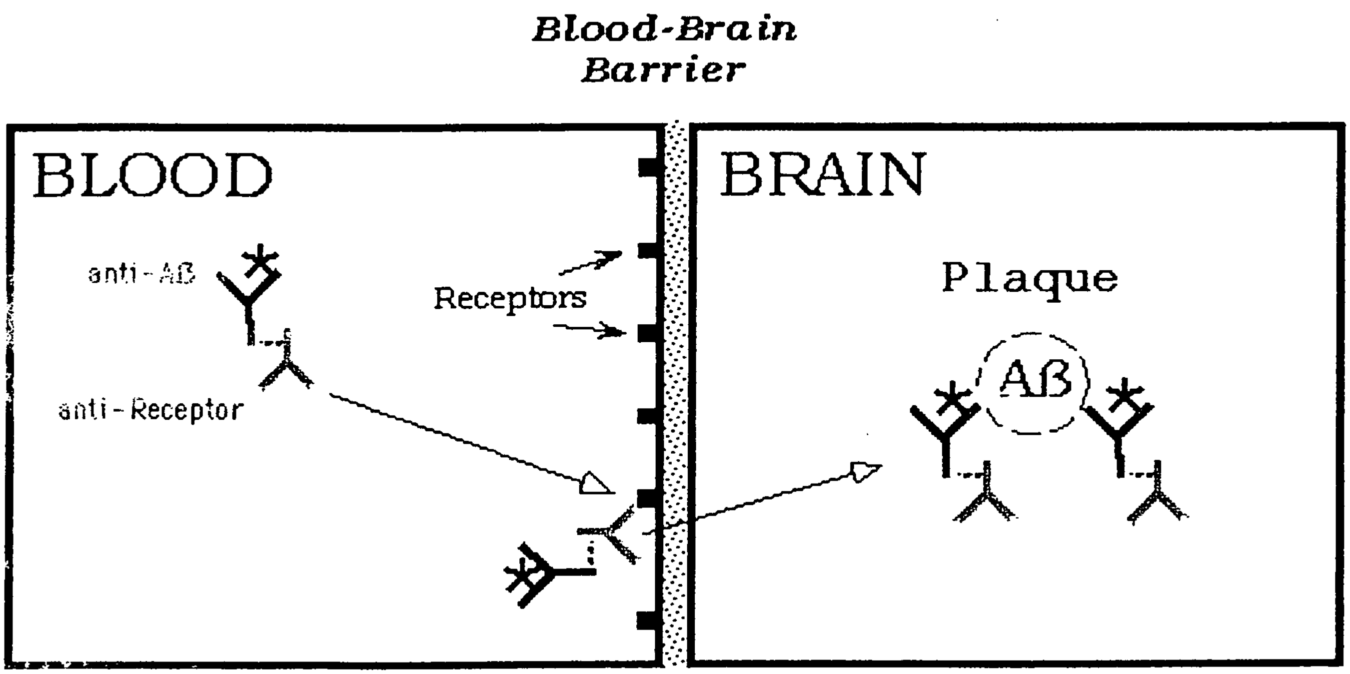


1 Transcytosis of Probe Across the Brain Capillary Endothelial

2 Specific Attachment of Radiolabeled Bifunctional Probe to Aß Plaque

2/7/98

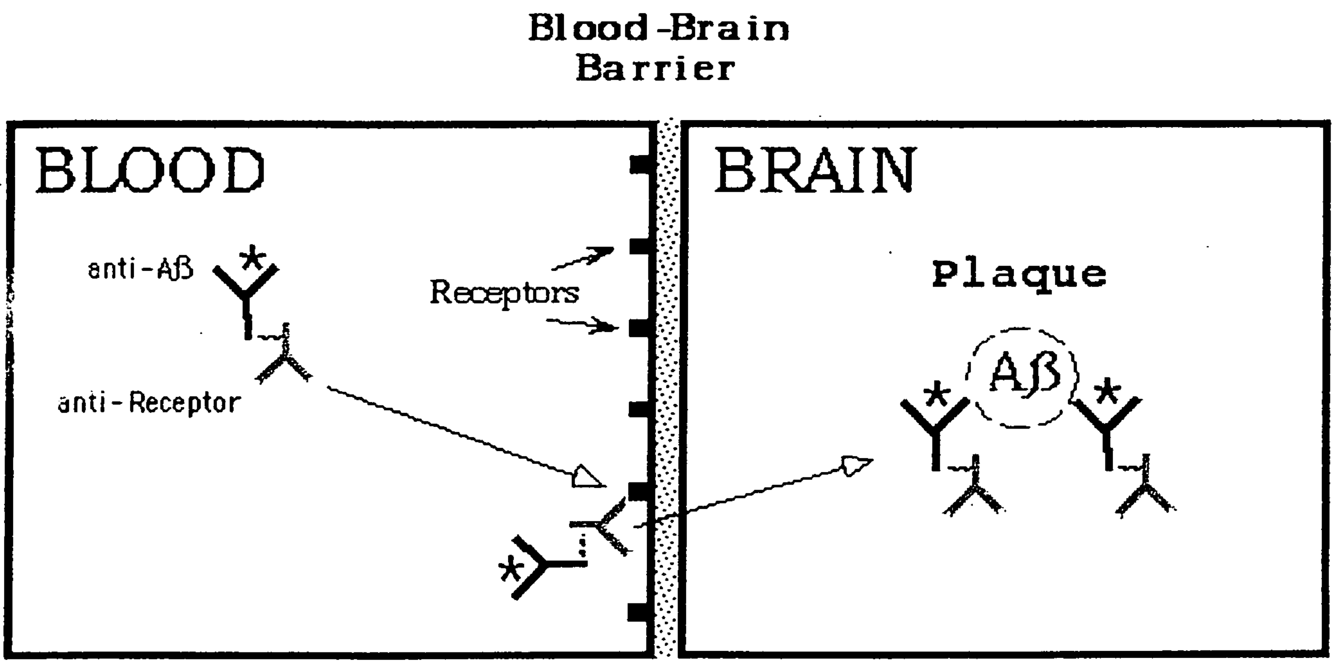
B001950



1 Transcytosis of Probe Across the Brain Capillary Endothelial

2 Specific Attachment of Radiolabeled Bifunctional Probe to Aß Plaque

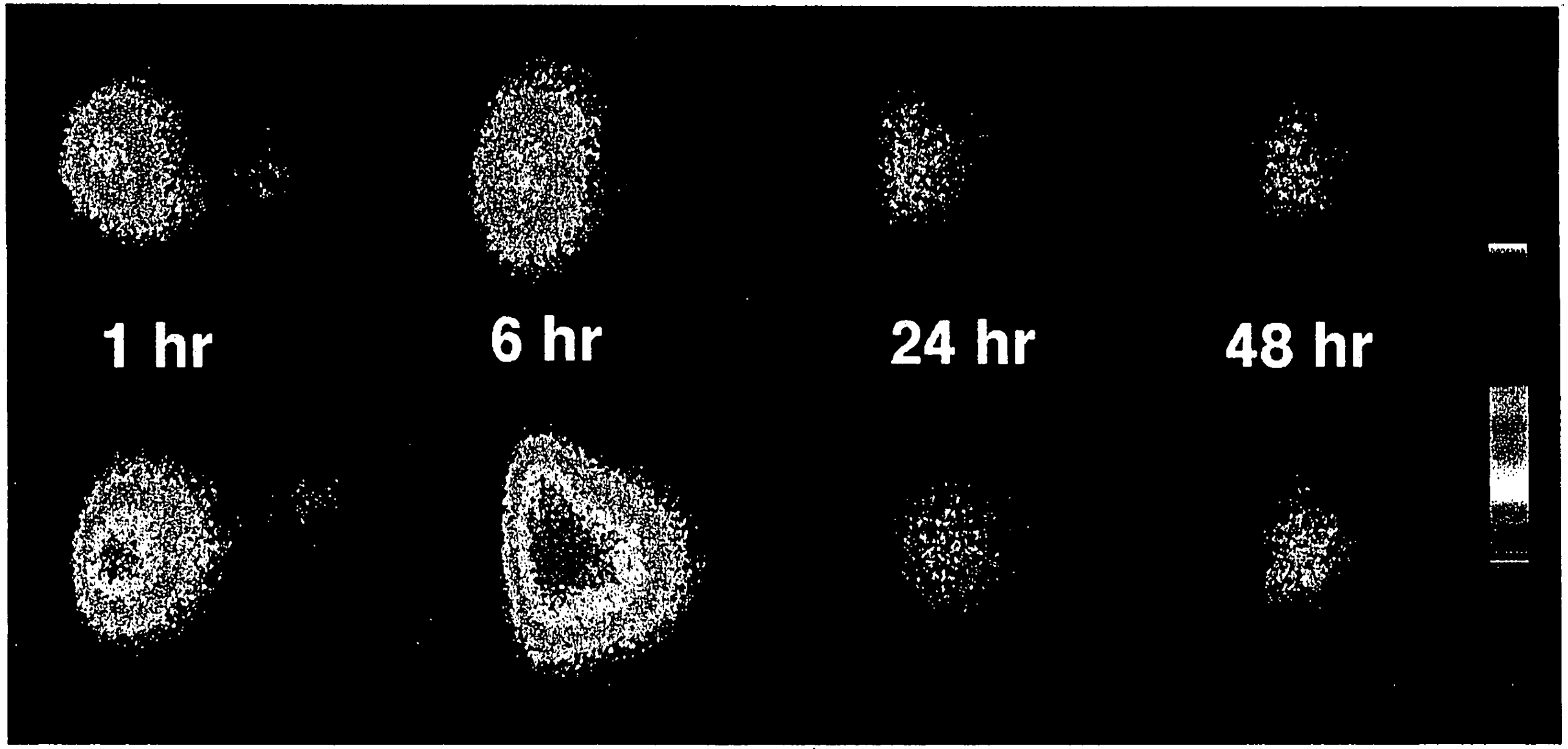
2/7/98



1 Transcytosis of Probe Across the Brain Capillary Endothelial

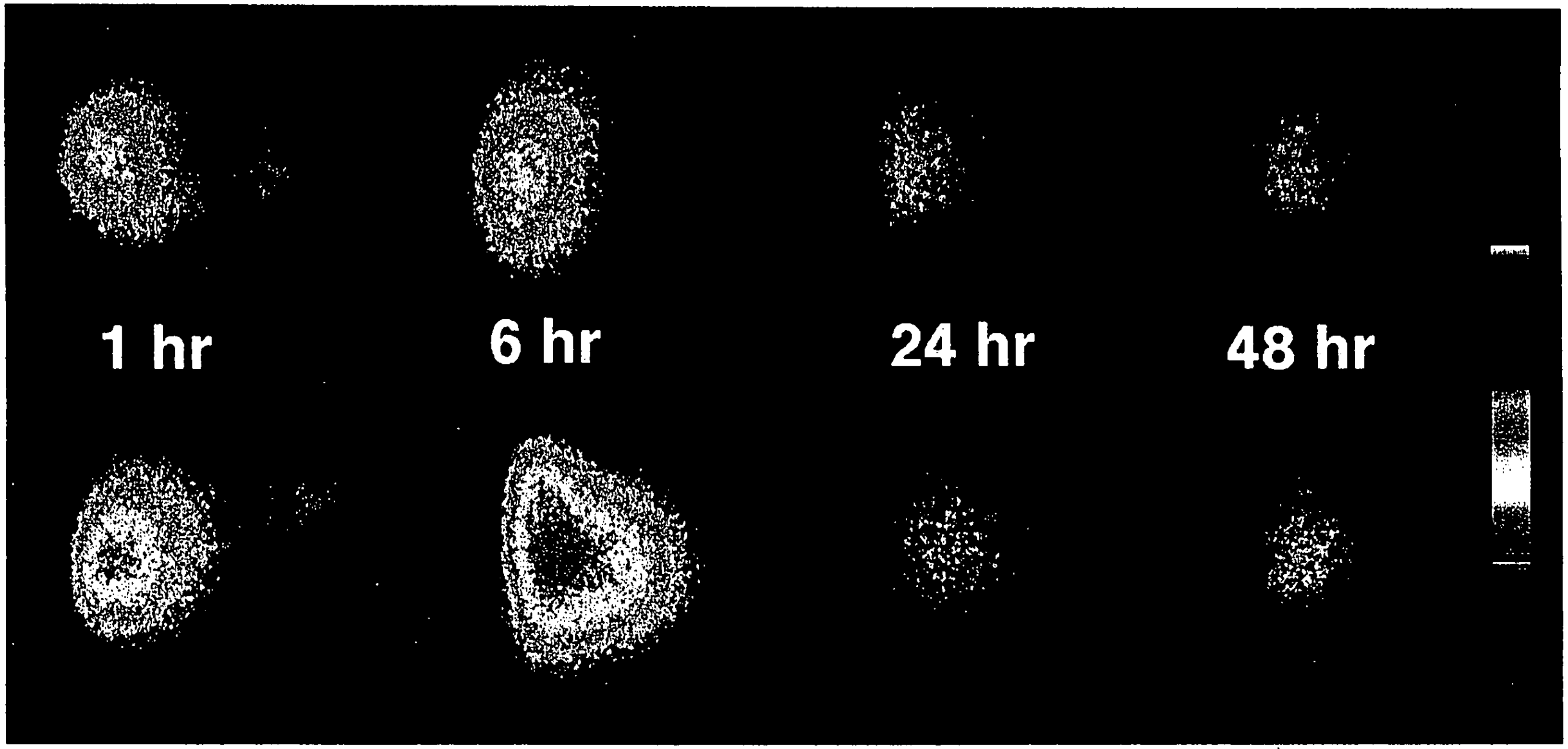
2 Specific Attachment of Radiolabeled Bifunctional Probe to Aß Plaque

2/17/98



1/18/08

B001953



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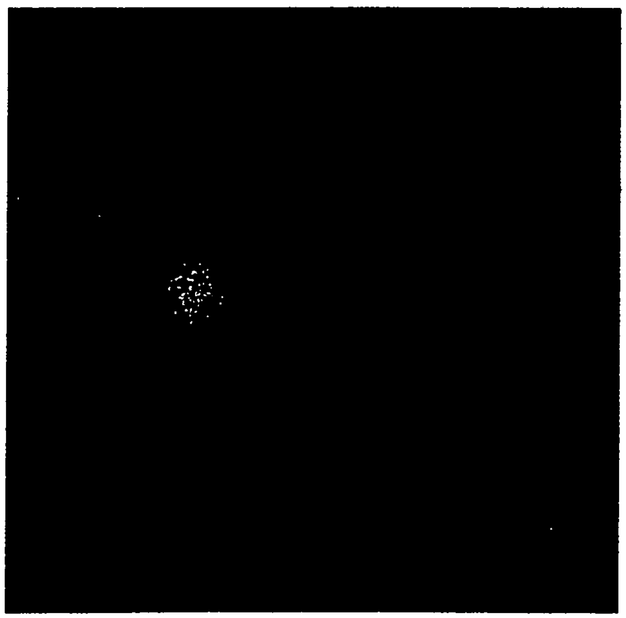
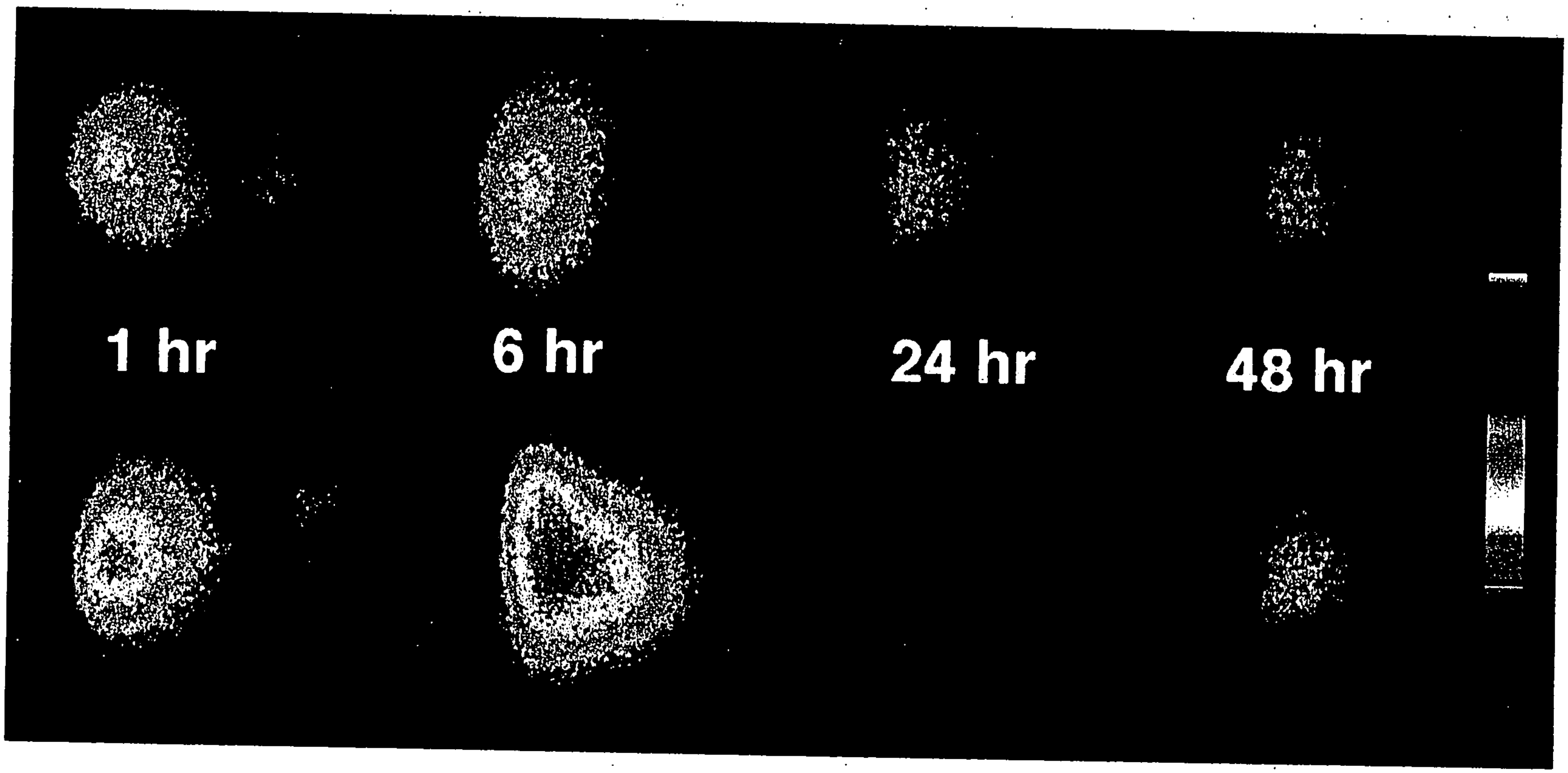
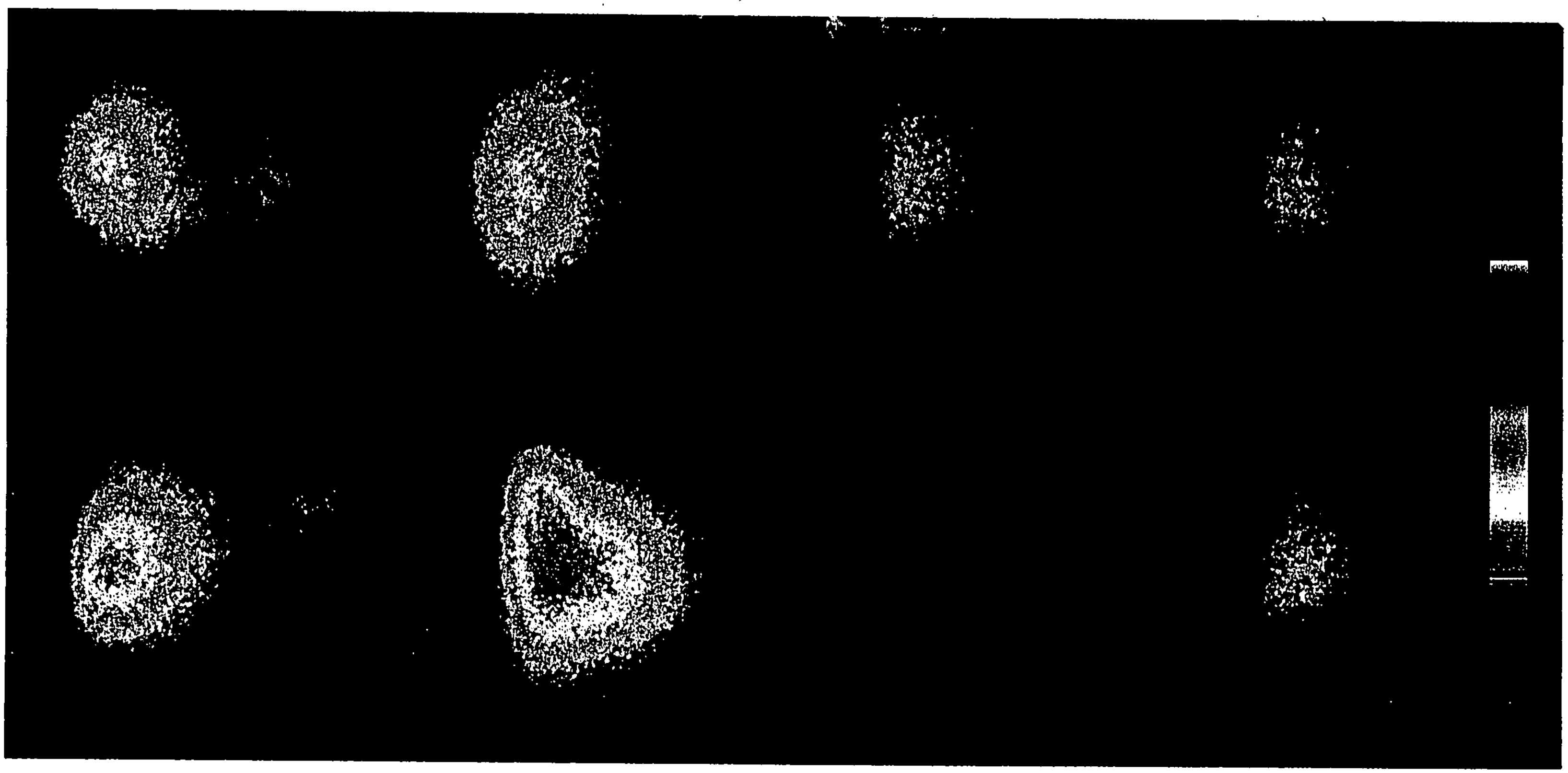


1/18/98



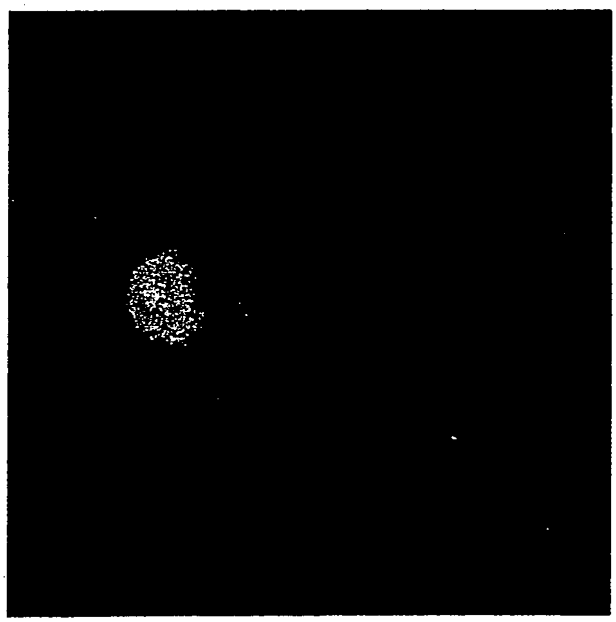
1/17/98

B001956

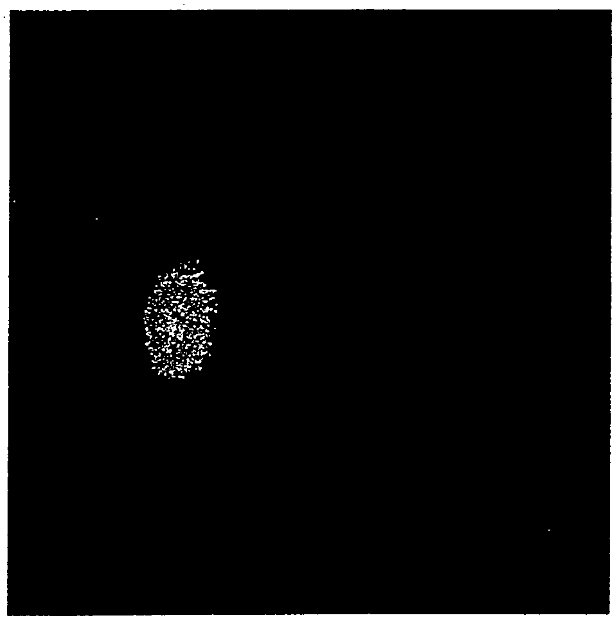


1/17/98

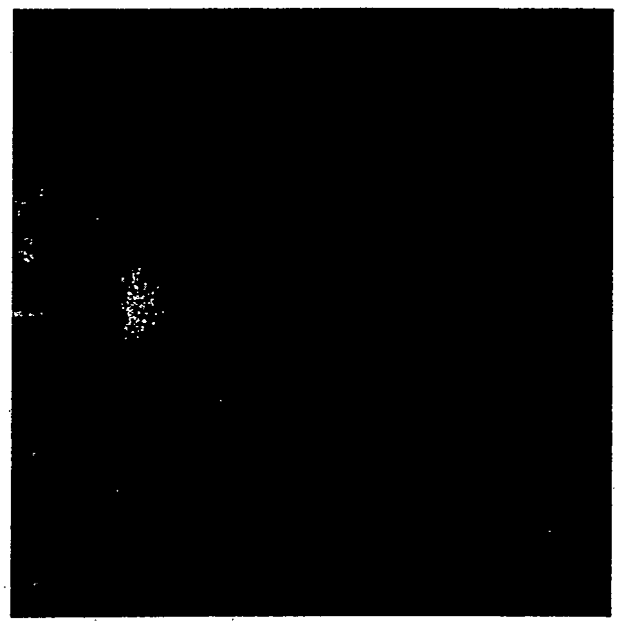
untitled 3 1/17/98
won't print



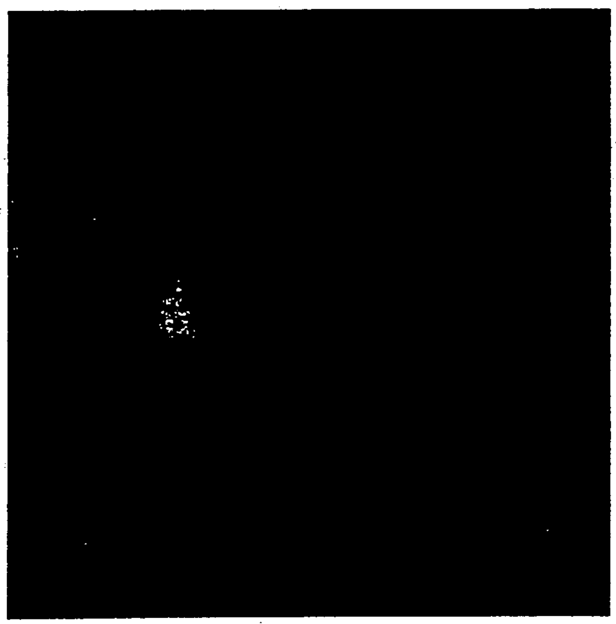
1/17/98



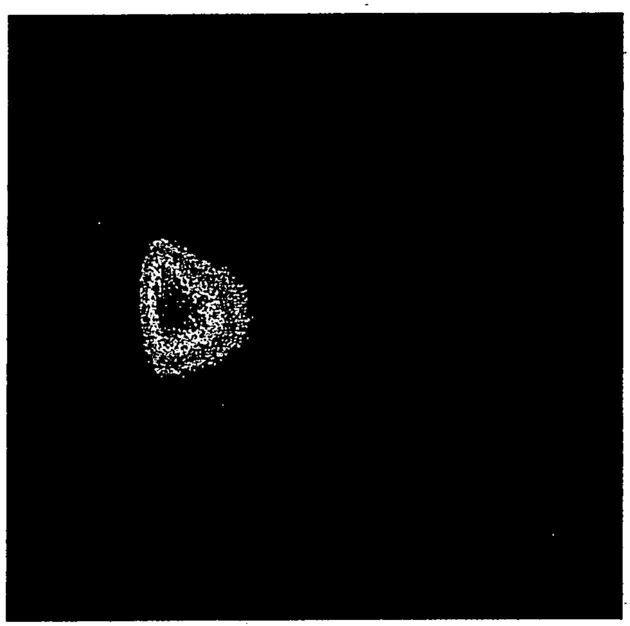
1/17/98



1/17/98



1/17/08



1/17/98

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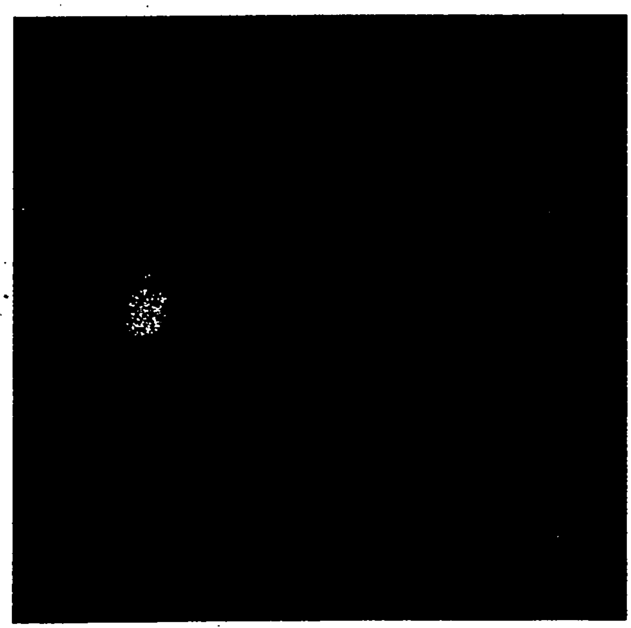
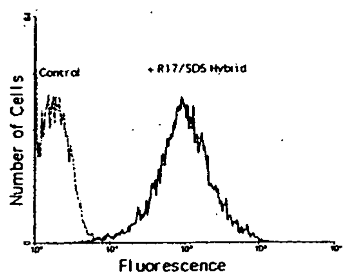
11/17/98

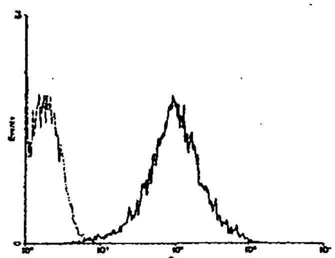


1/17/98

1/10/98



12/30/97



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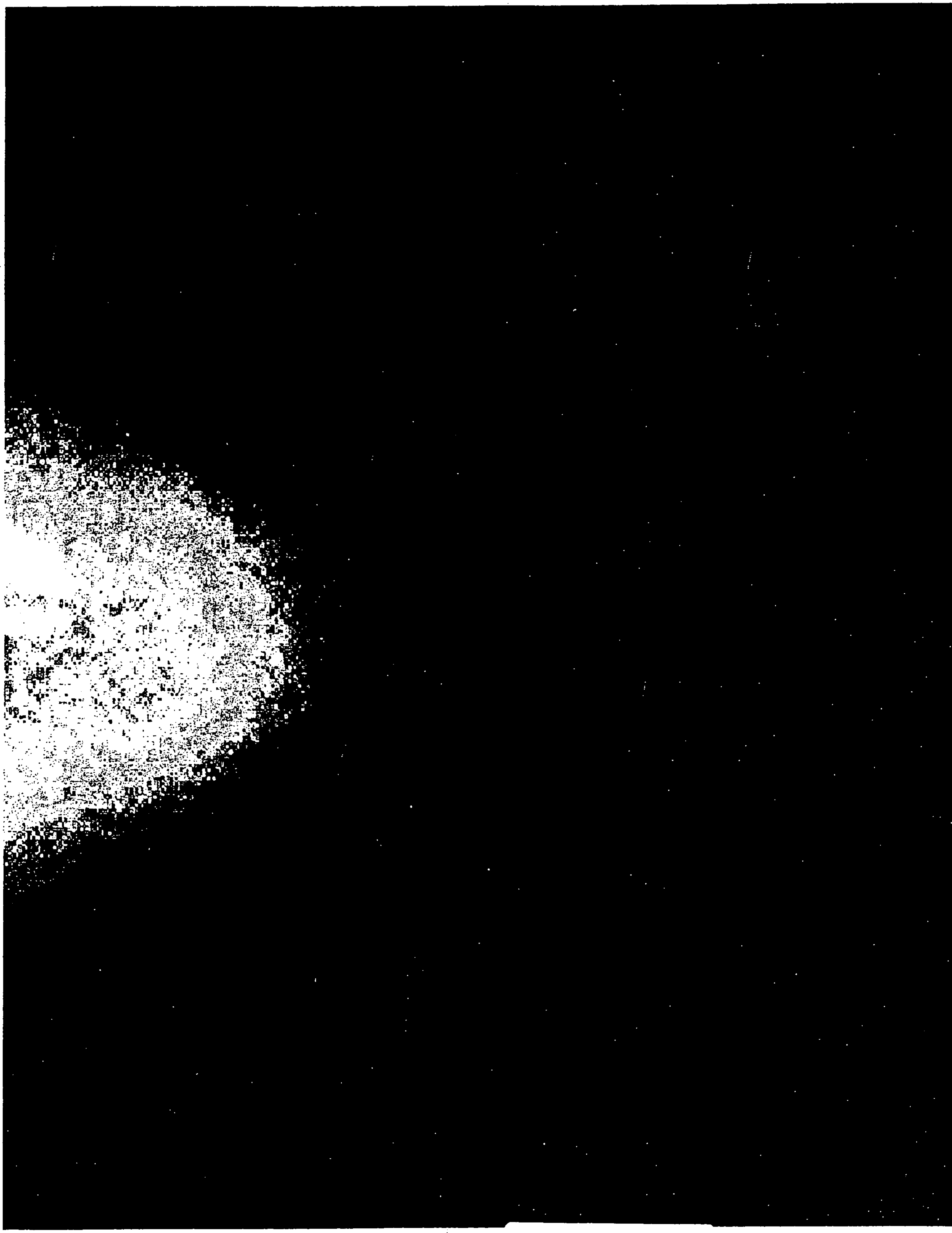

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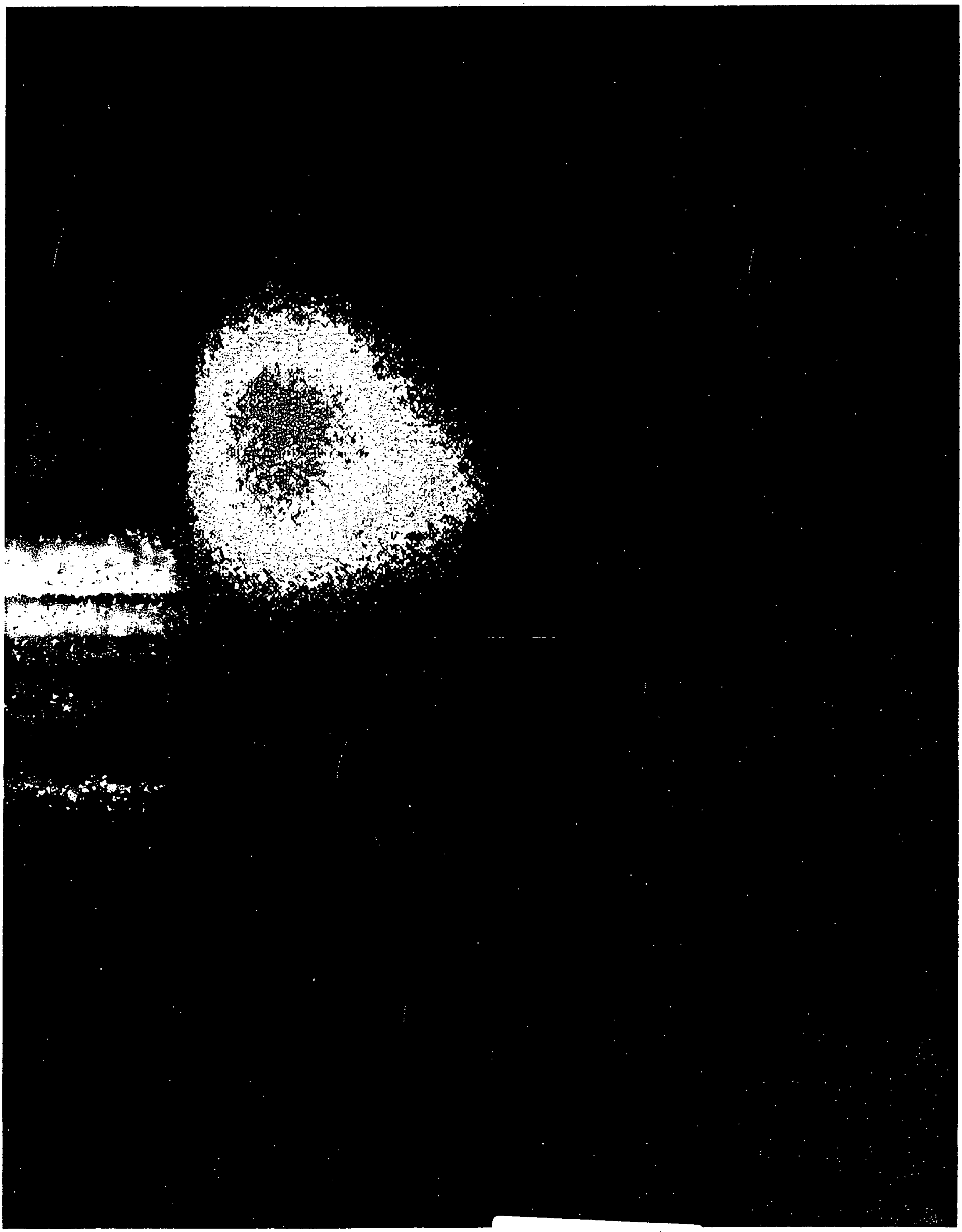

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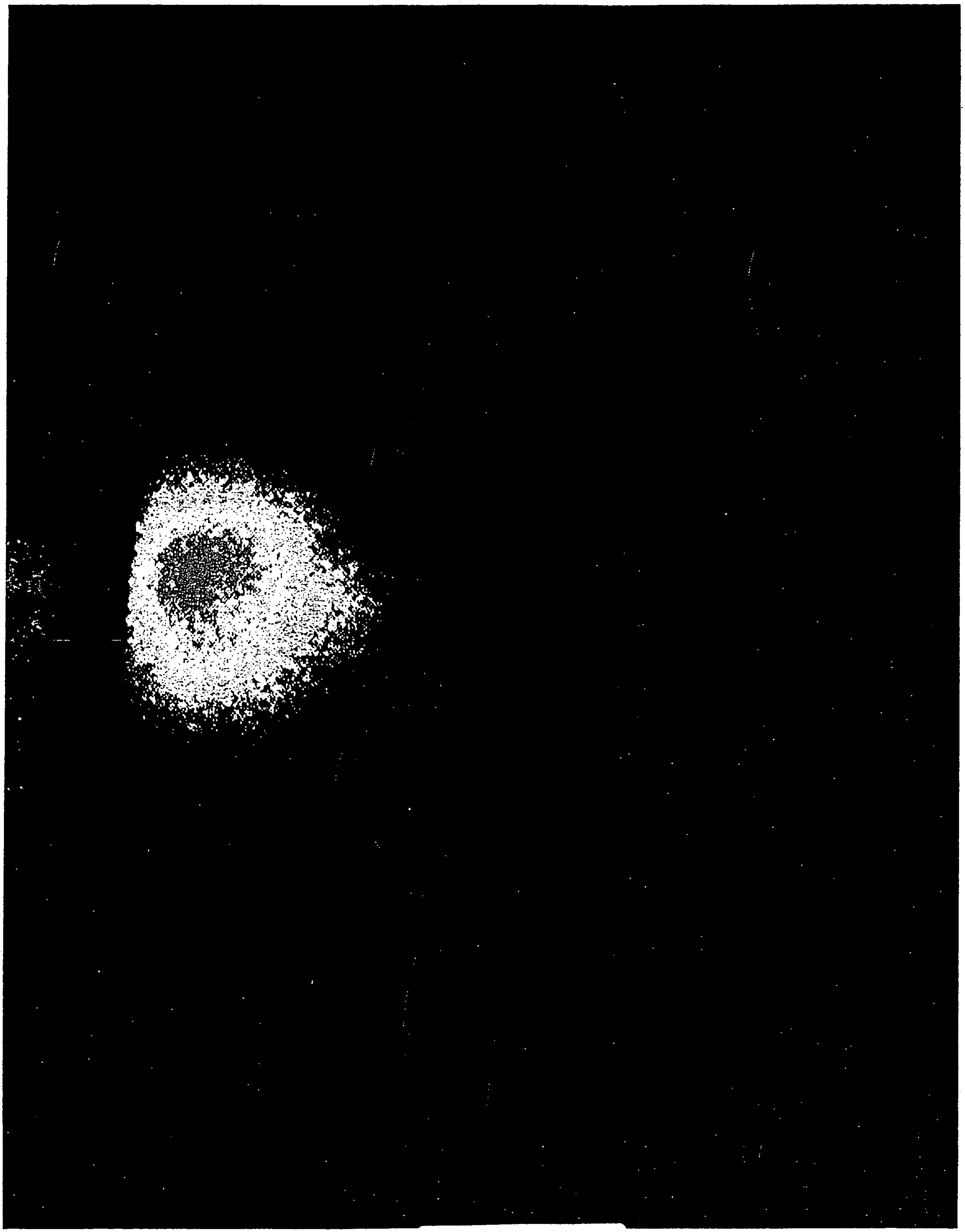

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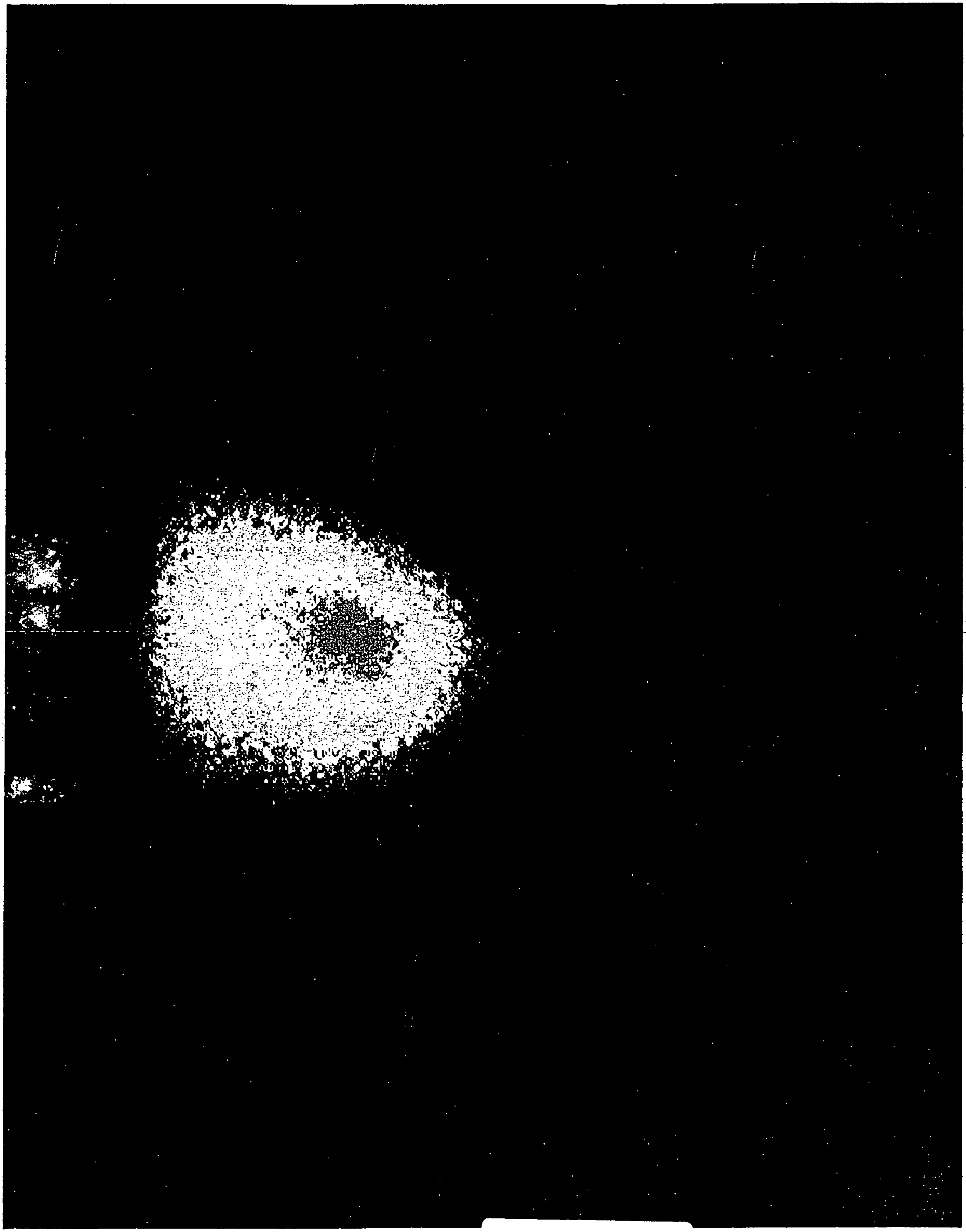
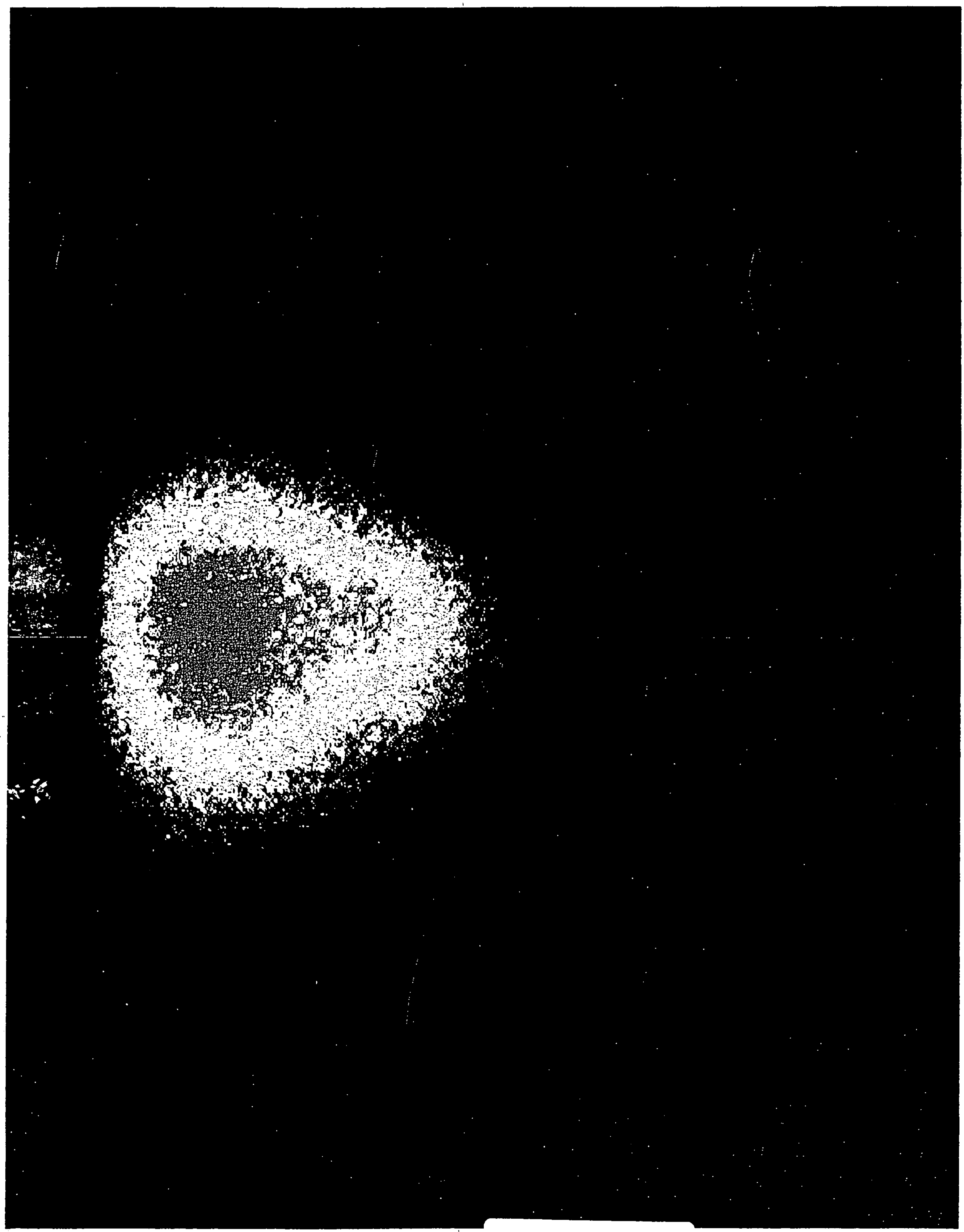
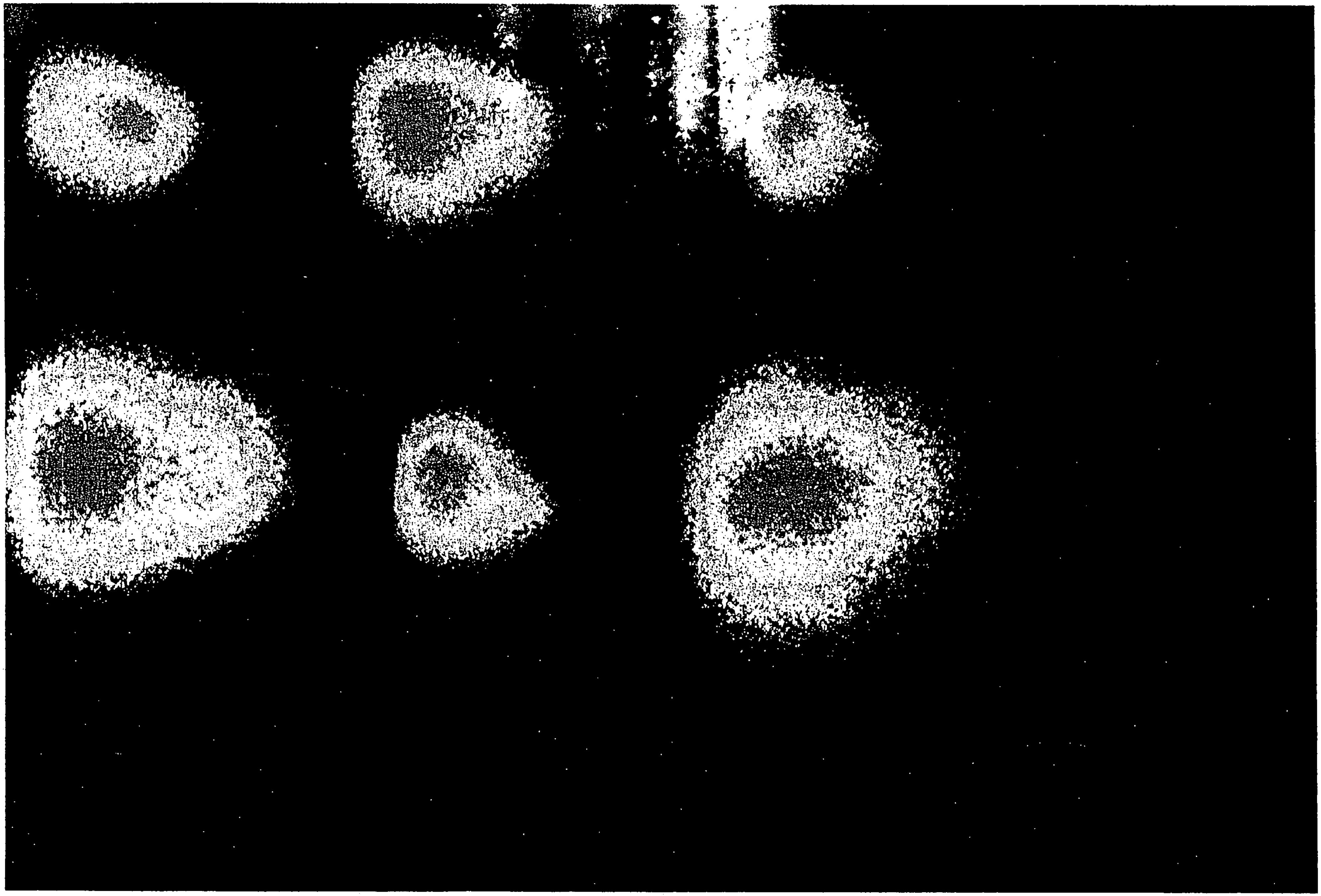
12/19/07





12/10/07



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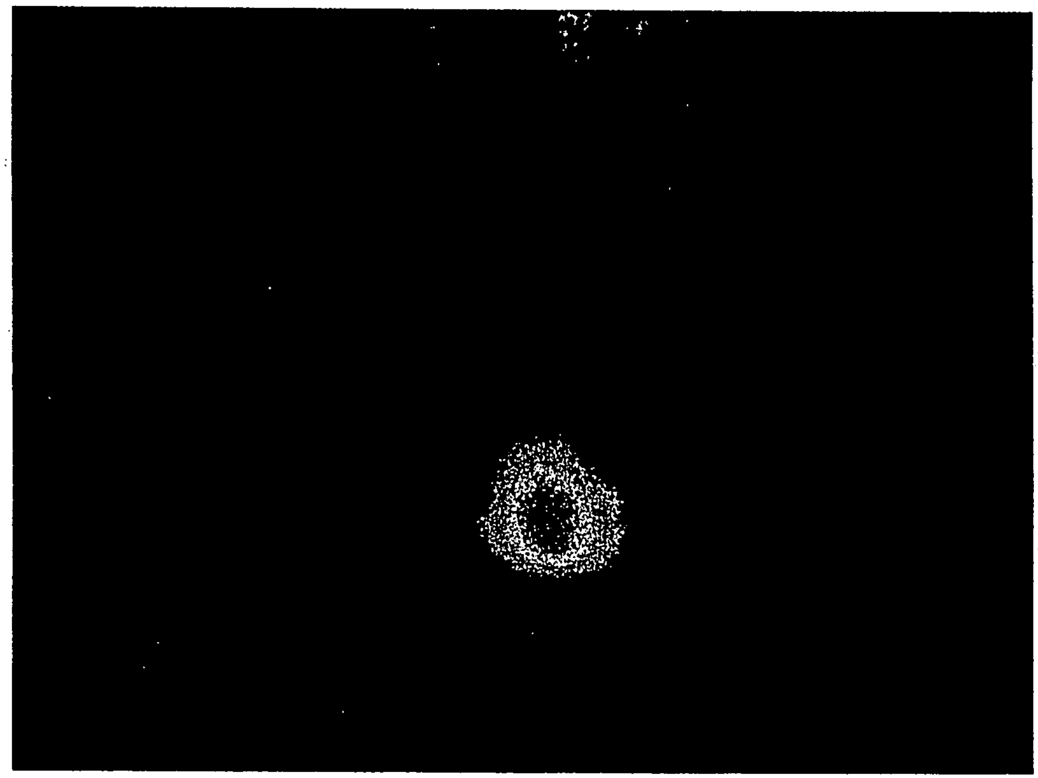

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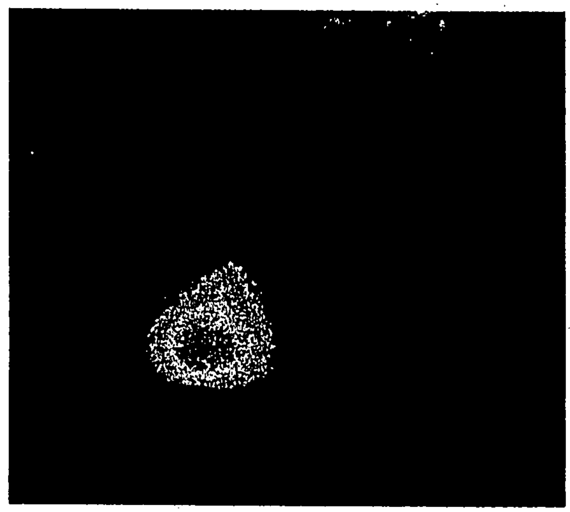

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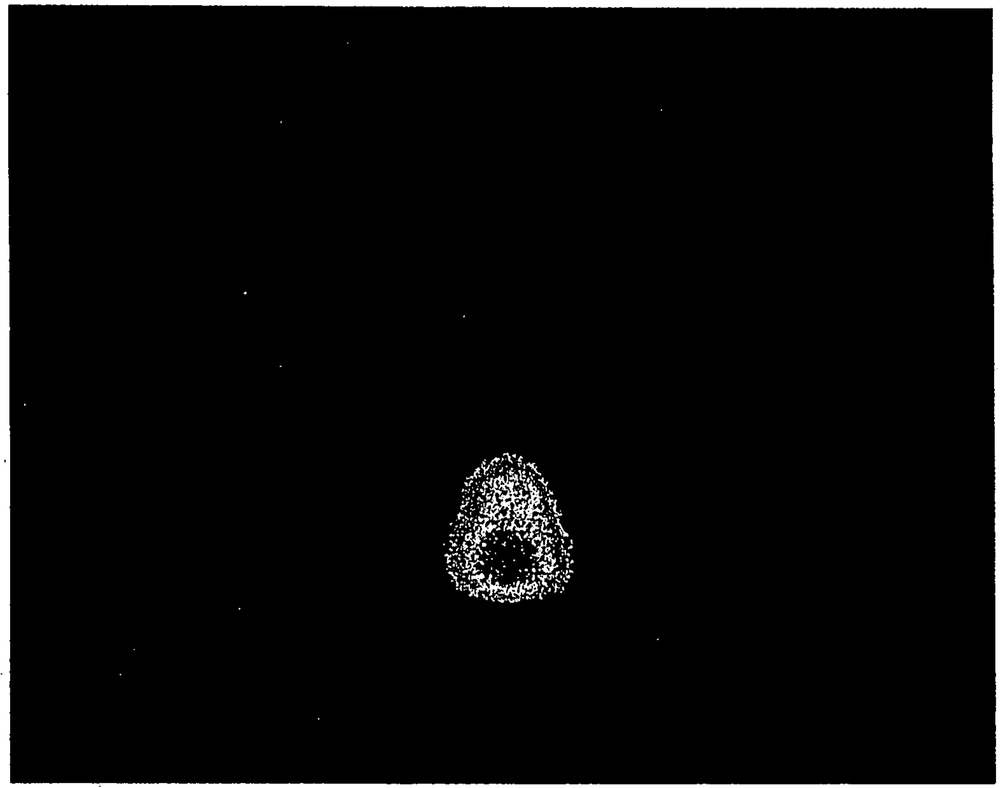

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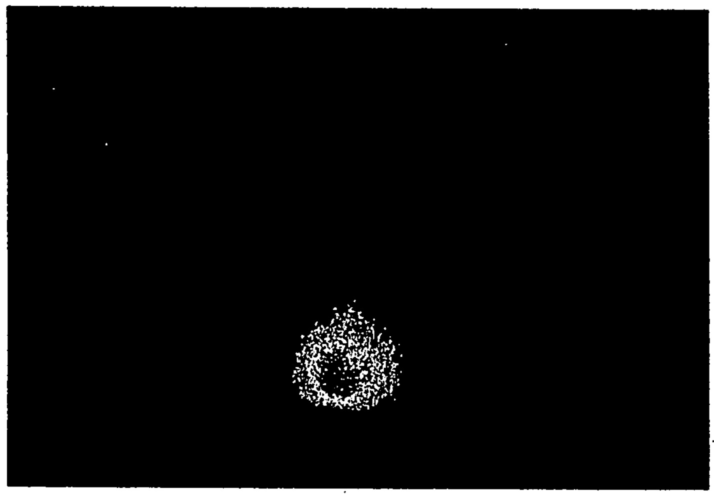
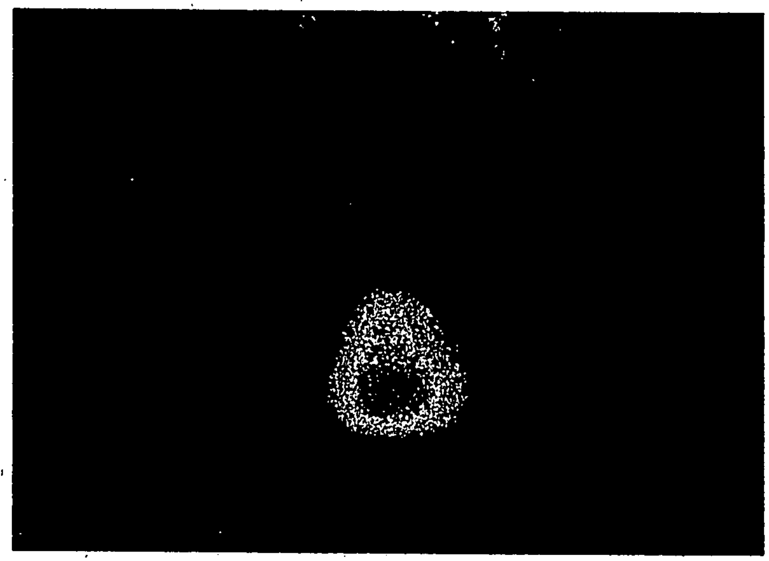


12/19/97



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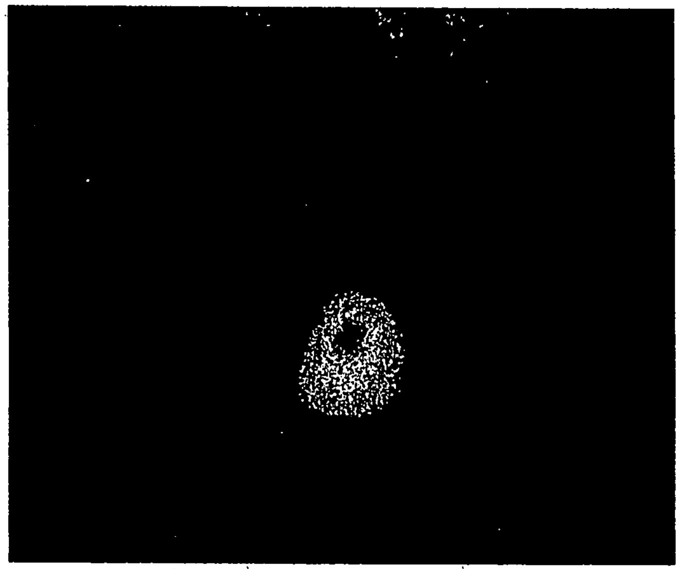

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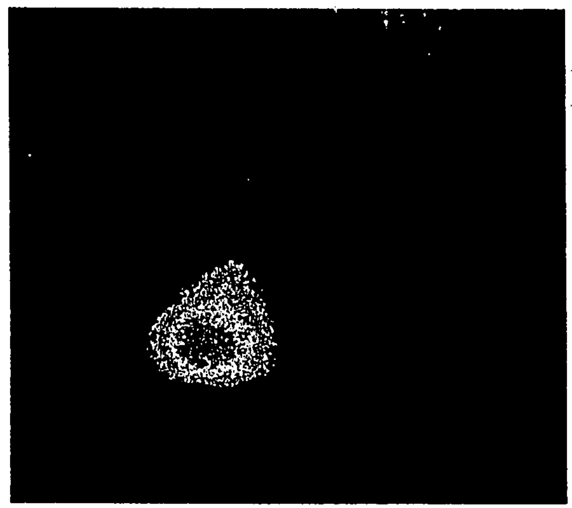
12/19/97



12/01/07

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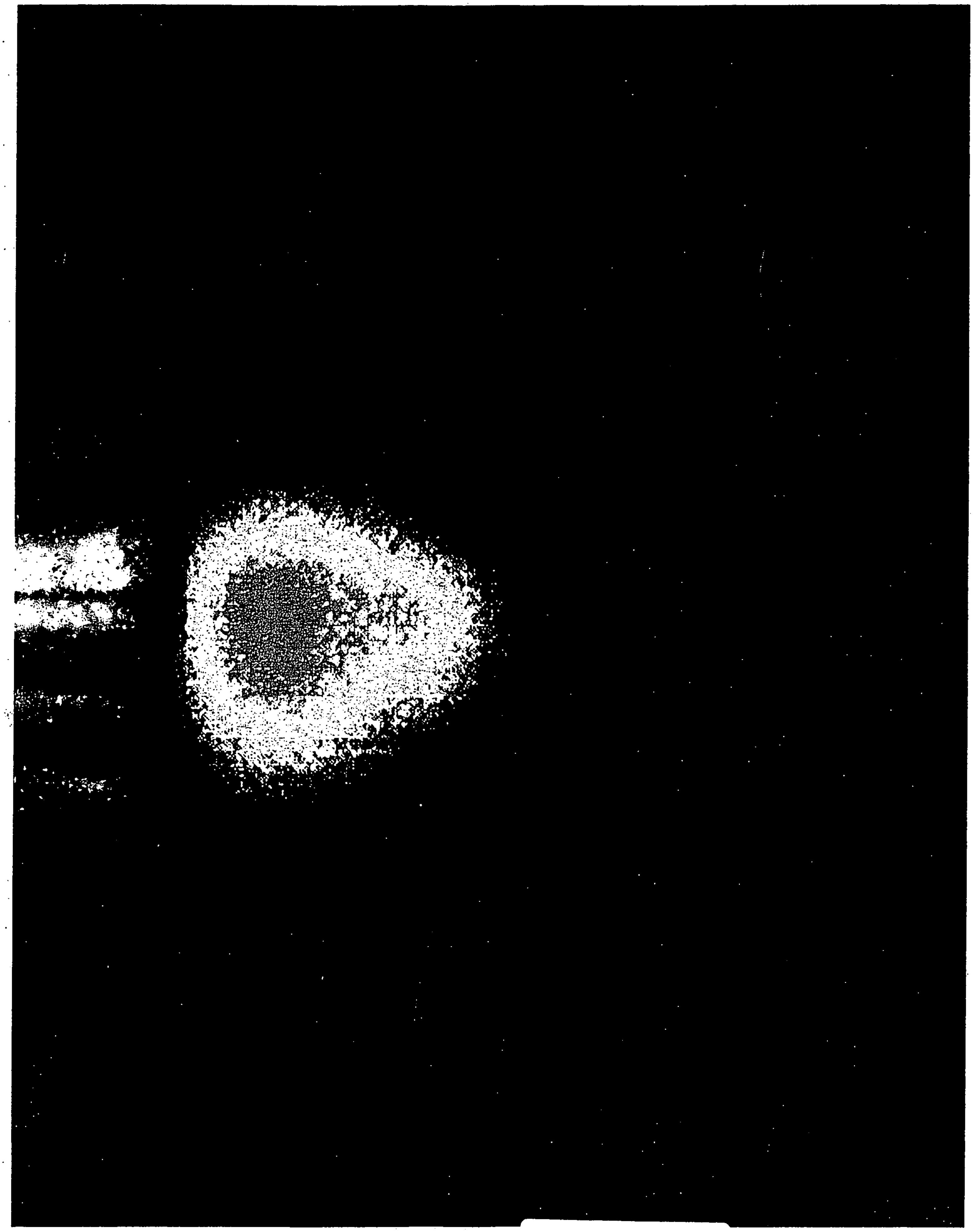

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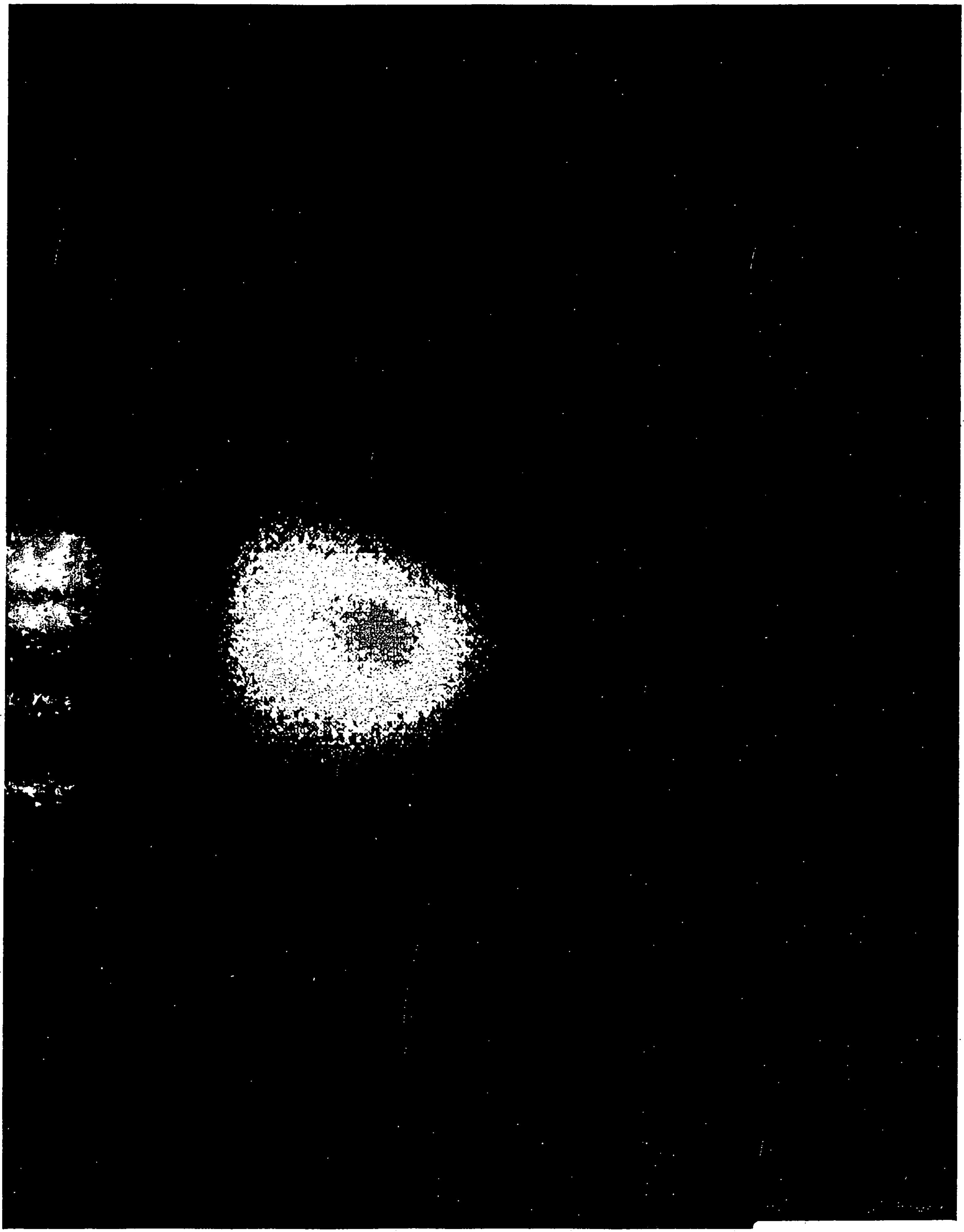
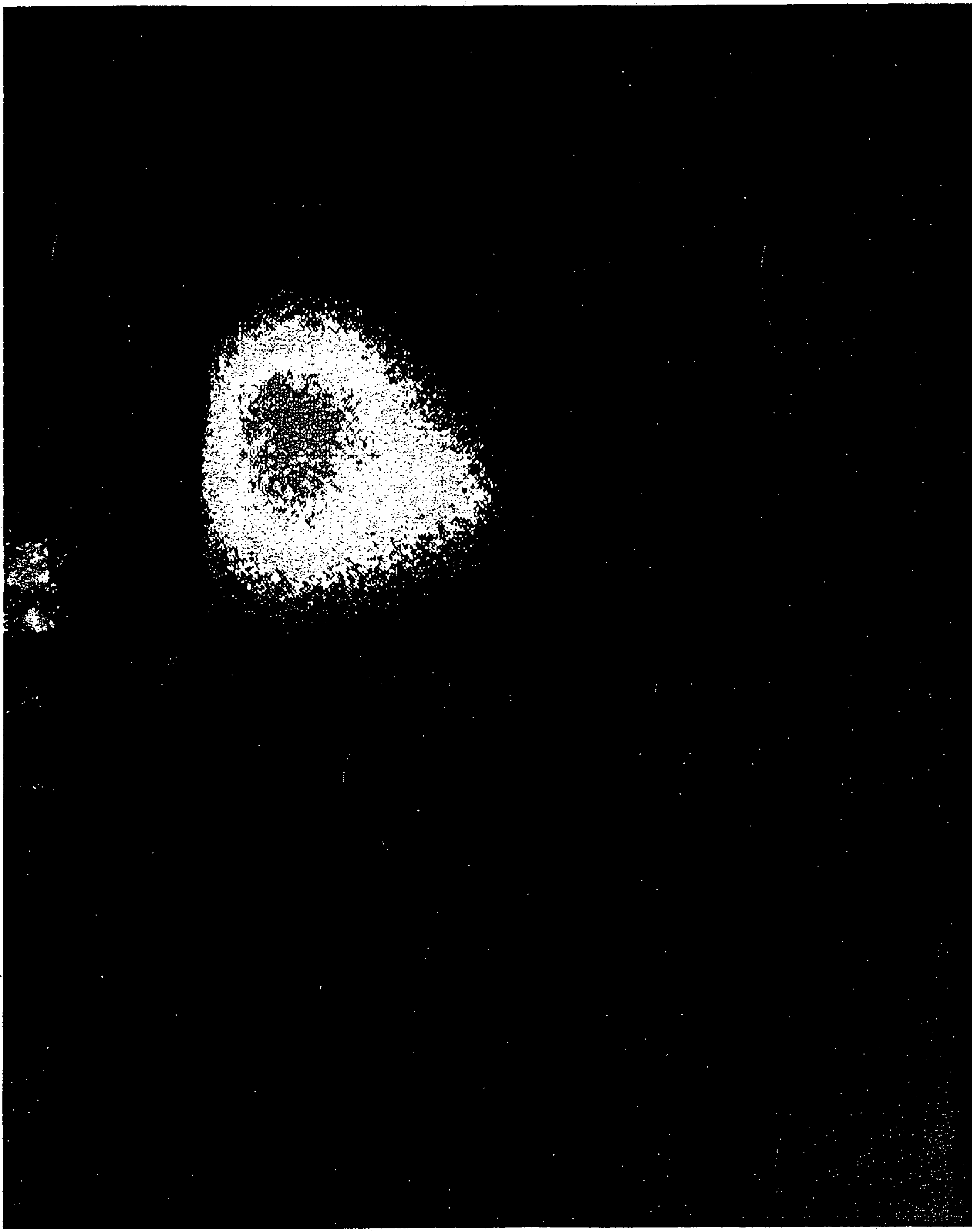


12/12/07 B001983

B001984

12/10/02



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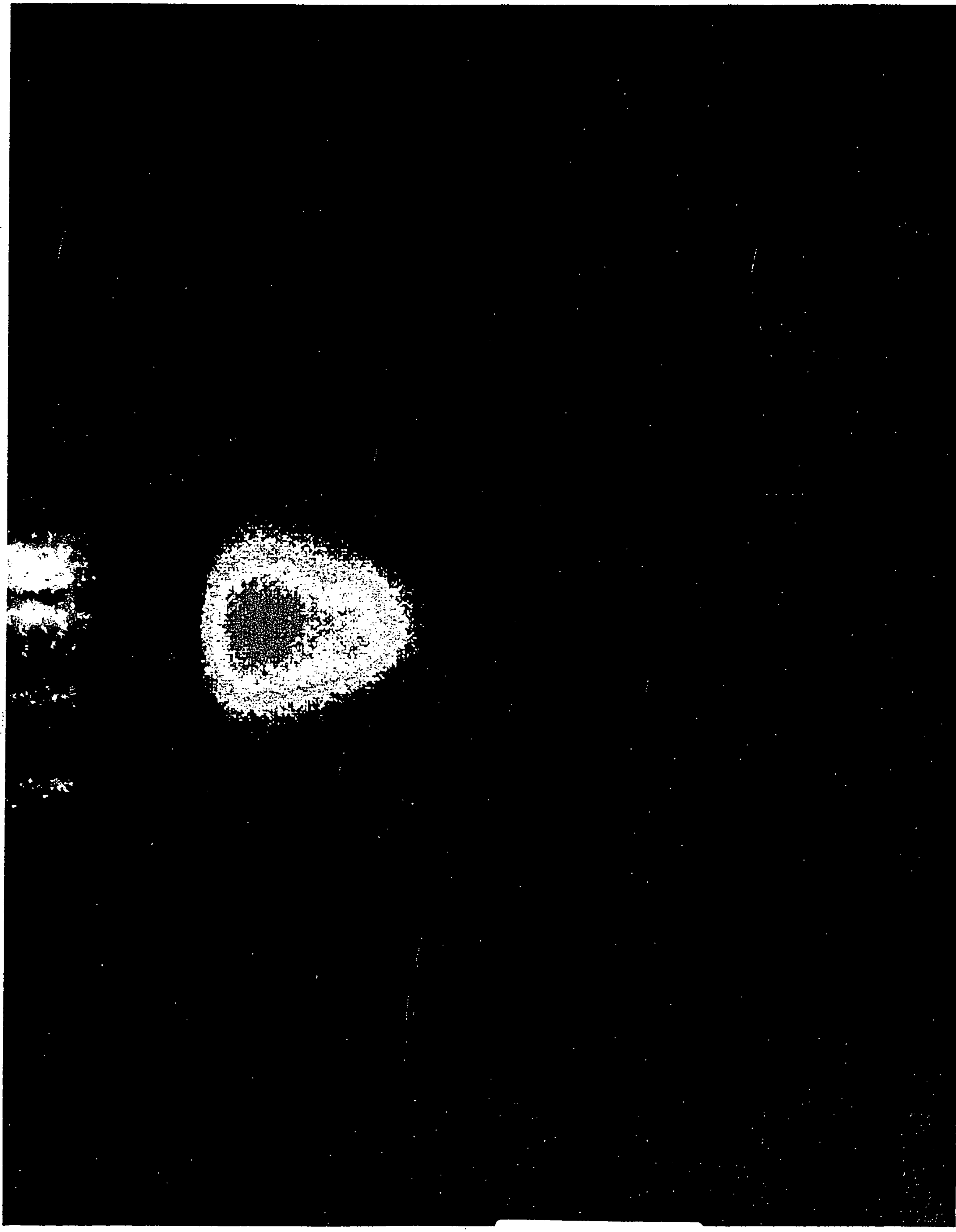

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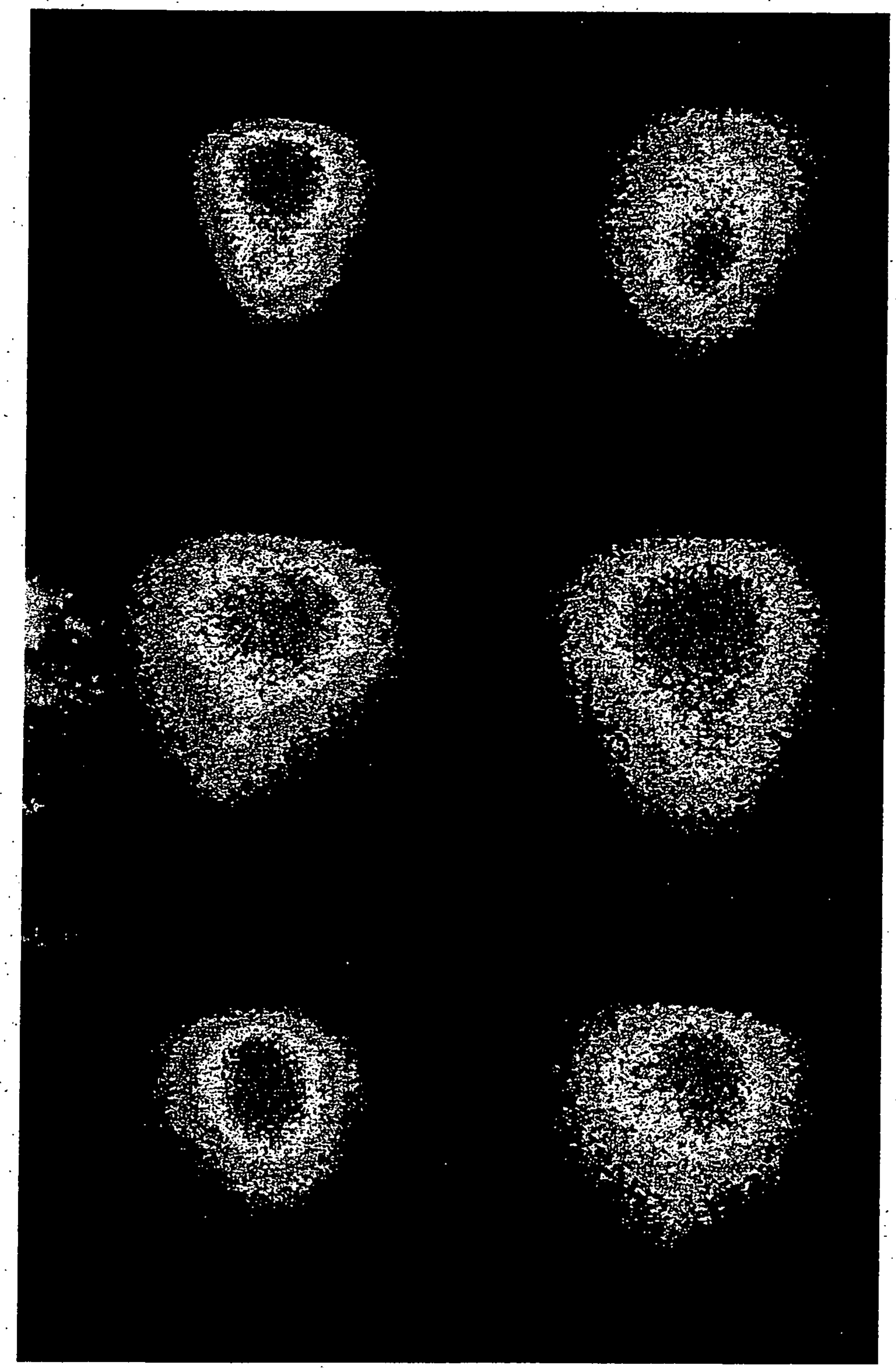

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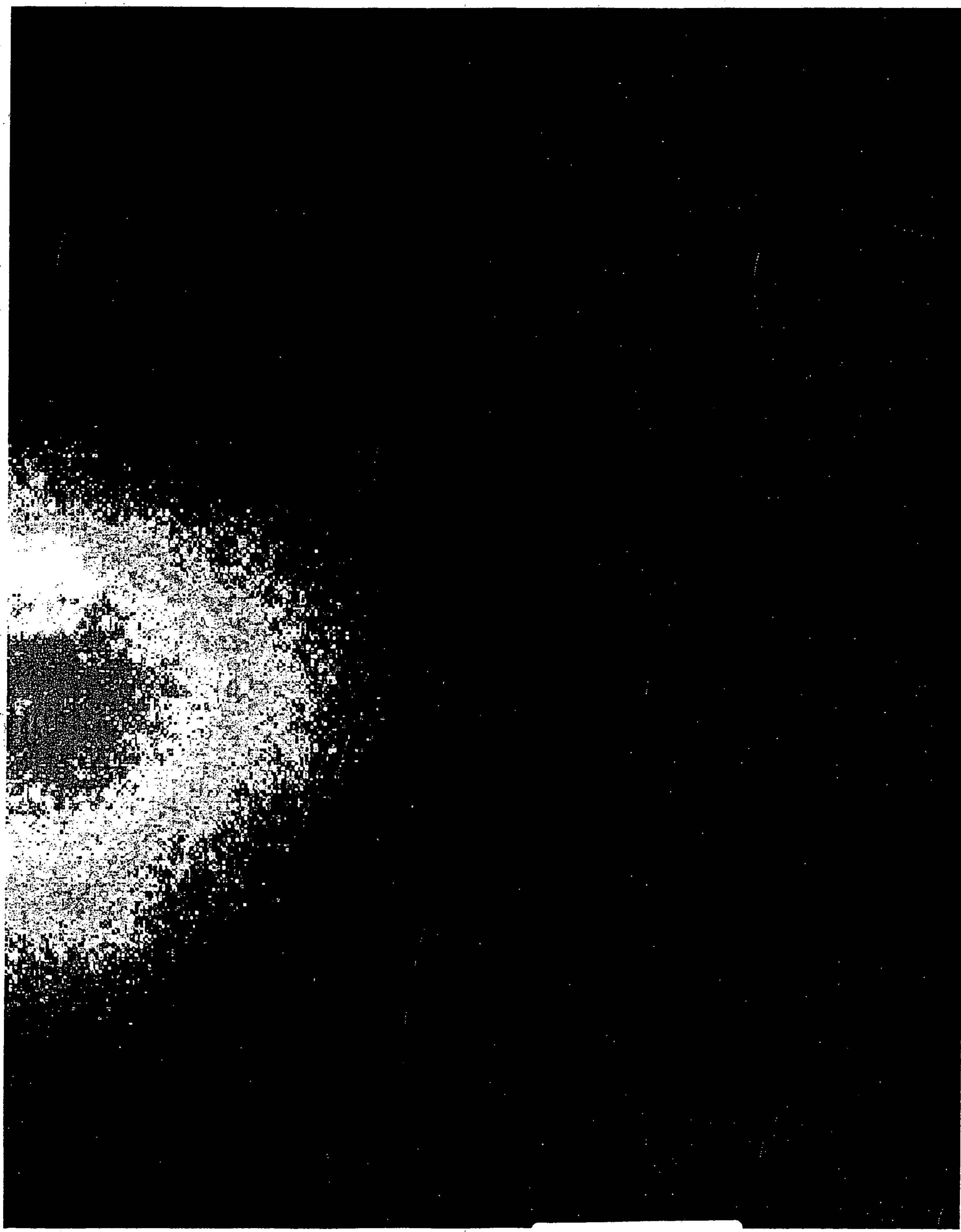

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12/10/07

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12/13/97

Created: Saturday, December 13, 1997 9:30 AM

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IAT 43
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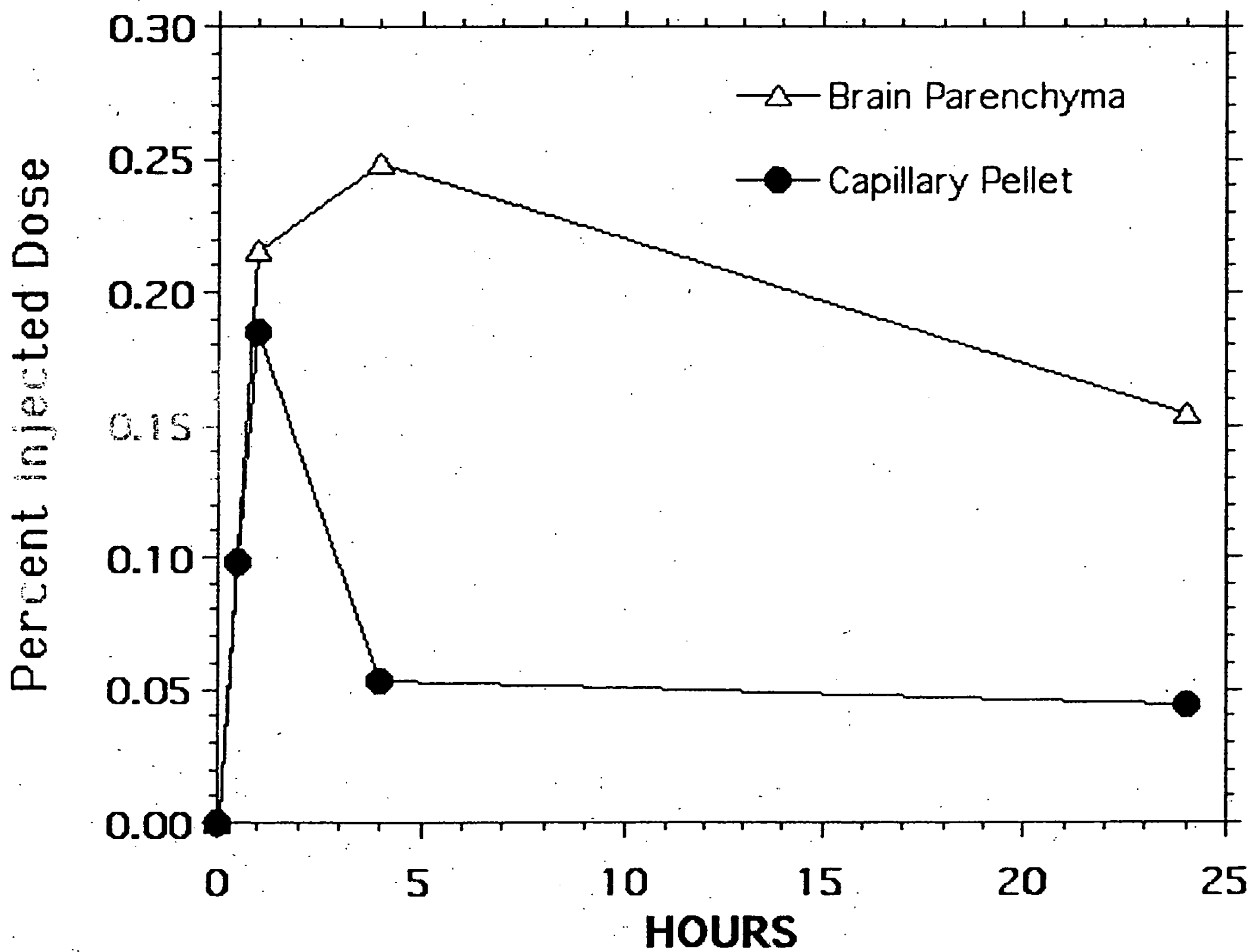
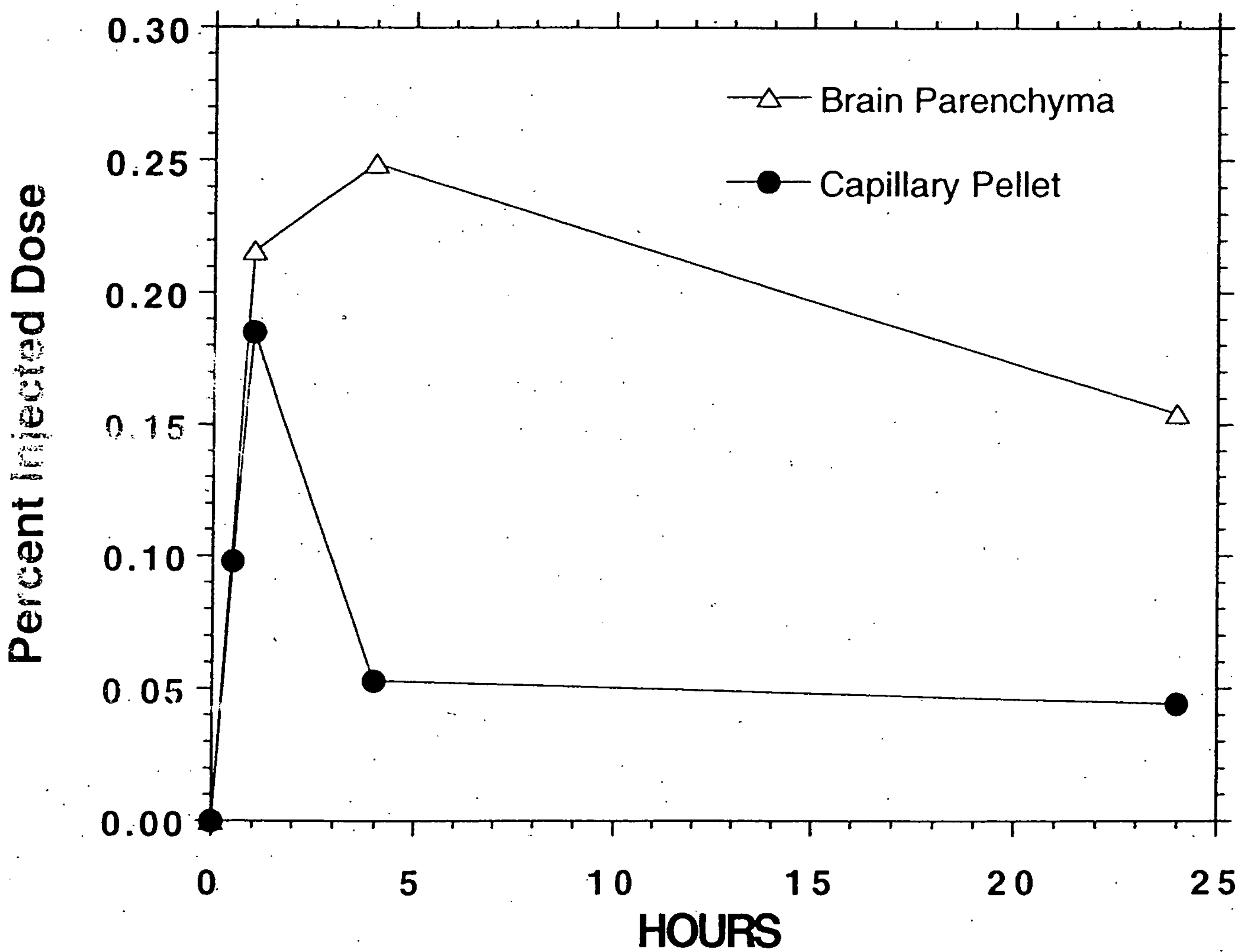


Brain Parenchyma
Capillary Pellet
Percent Injected Dose
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0
5
10
15
20
25
HOURS

12/10/97



0.30
0.25
0.20
0.15
0.10
0.05
0.00
Percent Injected Dose
Brain Parenchyma
Capillary Pellet
0
5
10
15
20
25
HOURS

12/10/97

H12 PS

	1	2	3	4	5	6	7	8	9	10	11	12
A	Hy	8E4 (1) 1g.f1.	8E4 (2) 1g.f1	8E6 +/- 1g.f1.	6F11 +/- 1g f1.	3B1 +/-	+ 4E6	+ 6E2	+ 4H9	+ 3B5	5A11 +/-	8E3 +/-
B	+ 6F11	6D12	+ 5A11	+/- 6F11	front 6E2	front 5G4						
C												
D												
E												
F												
G												
H												

injected 6E2
4H9 look bad
5A11 look bad

5G4
8E6
8D12
8E3
4G7

	1	2	3	4	5	6	7	8	9	10	11	12
1-H3 A	C 1:100	C 1:1000	C 1:10,000	6E2 1:100	6E2 1:1000	6E2 1:10,000	6E2 1:100,000					
B												
A12 PS C	C 1:100	C 1:1000	C 1:10,000	6E2 1:100	6E2 1:1000	6E2 1:10,000	6E2 1:100,000					
D												
E												
F												
G												
H												

Testing control + Ascites from 6E2 in serial dilutions on A12 PS plate + 1-H3 plate

Results:

C ⊖ } on Both plates
6E2 ⊕ }

PEG 4/2/96

1 PBS
2 PBS
3 Hy
4 Hy
5 5H11
6 2E3
7 5G11
8 2H11
9 1H2

1	1.00	25627	PBS
2	1.00	24159	
3	1.00	5163	Hy
4	1.00	4264	Hy
5	1.00	47871	5A11
6	1.00	15360	2E3
7	1.00	7682	5G11
8	1.00	8818	2H11
9	1.00	5891	1H2
10	1.00	138	

B001453

Date: Wed, May 8, 1996 10:40 AM
Data: 0-40%-08MAY96-001

Sample: FITC
100%B
C8 column analytical
Detector prog #1 Channel a: 320 nm
Channel b: 340 nm

2010.4
2041.3
2059.5
2083.5
2209.9
2252.7

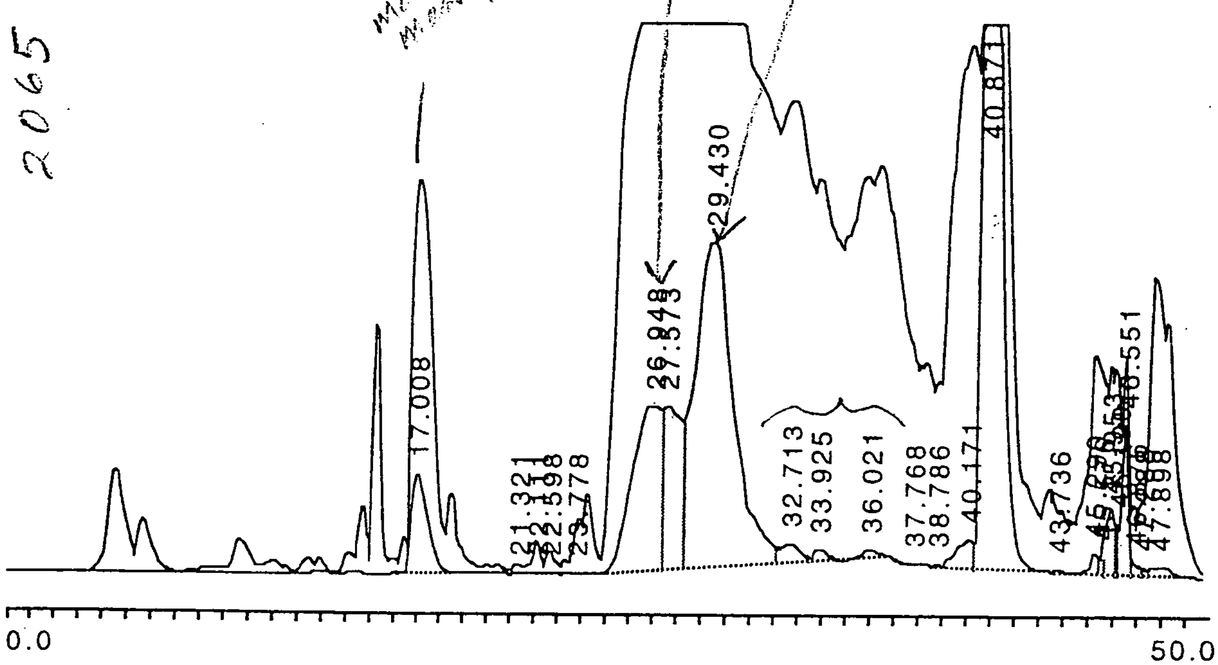
Processing File:
Method: 0-40%
Inject Vol: 0.01
Sampling Int: 0.1 Seconds

2062.
2105.6
2251.4
2295.6

Data:

nothing in M. mapper

Need
2021
2109
2065



Analysis: Channel B

Peak No.	Time	Type	Height(μV)	Area(μV-sec)	Area%
1	17.008	N	172209	7759950	4.318
2	21.321	N	2240	34204	0.019
3	22.111	N1	2484	26080	0.014
4	22.598	N2	613	10161	0.005
5	23.778	N	1171	23120	0.012
6	26.948	N1	291113	23090218	12.851
7	27.573	N2	289484	14900296	8.292
8	29.430	N3	572788	61421414	34.184
9	32.713	N4	31394	1877428	1.044
10	33.925	N5	19989	610259	0.339

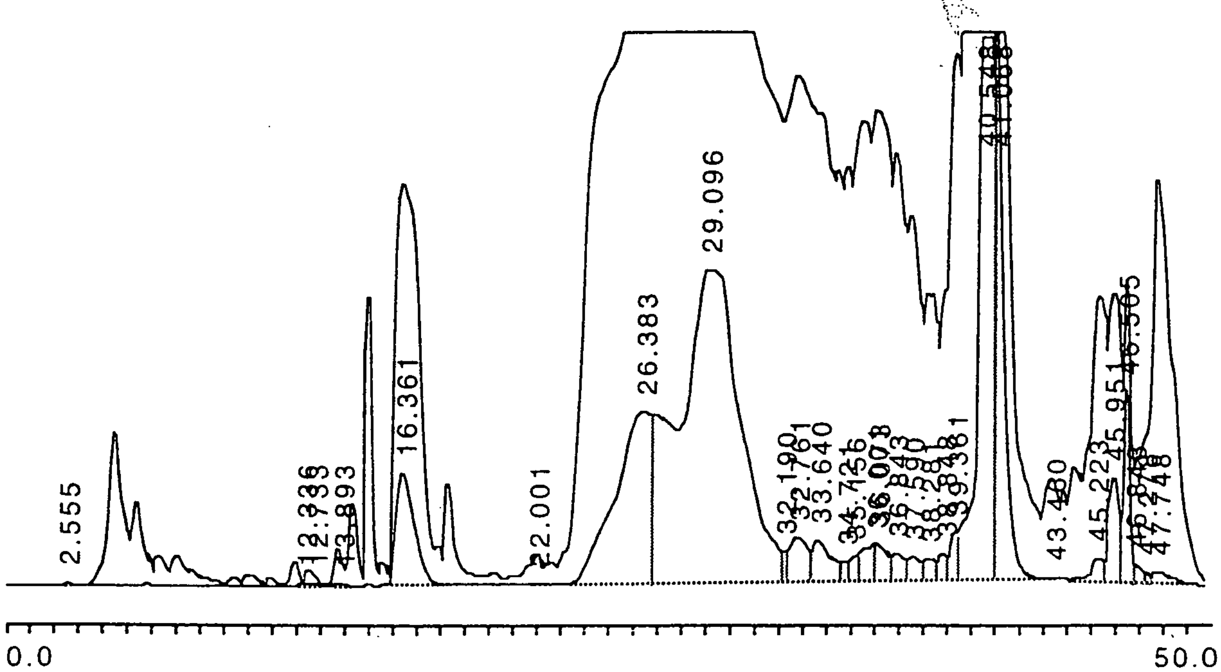
Date: Wed, May 8, 1996 2:46 PM
Data: 0-40%-08MAY96-002

Sample: FITC
100%B
C8 column analytical
Detector prog #1 Channel a: 320 nm
Channel b: 340 nm

Processing File:
Method: 0-40%
Inject Vol: 0.01
Sampling Int: 0.1 Seconds

Note this didn't dissolve well in H_2O? complex?? complexed

Data:



Analysis: Channel B

Peak No.	Time	Type	Height(µV)	Area(µV-sec)	Area%
1	2.555	N	1845	14477	0.006
2	12.336	N1	2247	46972	0.020
3	12.733	N2	2073	31698	0.013
4	13.893	N	1600	36210	0.015
5	16.361	N	196685	9232468	4.000
6	22.001	N1	1524	12596	0.005
	22.406	N2	120	1263	0.000
7	26.383	N1	302576	29206006	12.654
8	29.096	N2	554206	96473482	41.801
9	32.190	N3	54243	764307	0.331

Date: Wed, May 8, 1996 2:46 PM
Data: 0-40%-08MAY96-002

Analysis: Channel B

Peak No.	Time	Type	Height(µV)	Area(µV-sec)	Area%
10	32.761	N4	77228	3759671	1.629
11	33.640	N5	70339	3750029	1.624
12	34.721	N6	33176	673586	0.291
13	35.156	N7	42385	808514	0.350
14	36.001	N8	58970	2264899	0.981
15	36.073	N9	63433	2167327	0.939
16	36.843	N10	47397	1547063	0.670
17	37.590	N11	38097	1359025	0.588
18	38.281	N12	34917	1238935	0.536
19	38.848	N13	43421	1026684	0.444
20	39.361	N14	80307	1789712	0.775
21	40.548	N15	953400	42886366	18.582
22	41.068	N16	963899	19119218	8.284
23	43.460	N17	1287	33108	0.014
24	45.223	N1	39296	1275709	0.552
25	45.951	N2	181947	4919475	2.131
26	46.505	N3	336828	4476355	1.939
27	46.843	N4	29840	589570	0.255
28	47.278	N5	17736	244386	0.105
29	47.748	N6	20709	1040318	0.450
Total Area				230789429	99.984

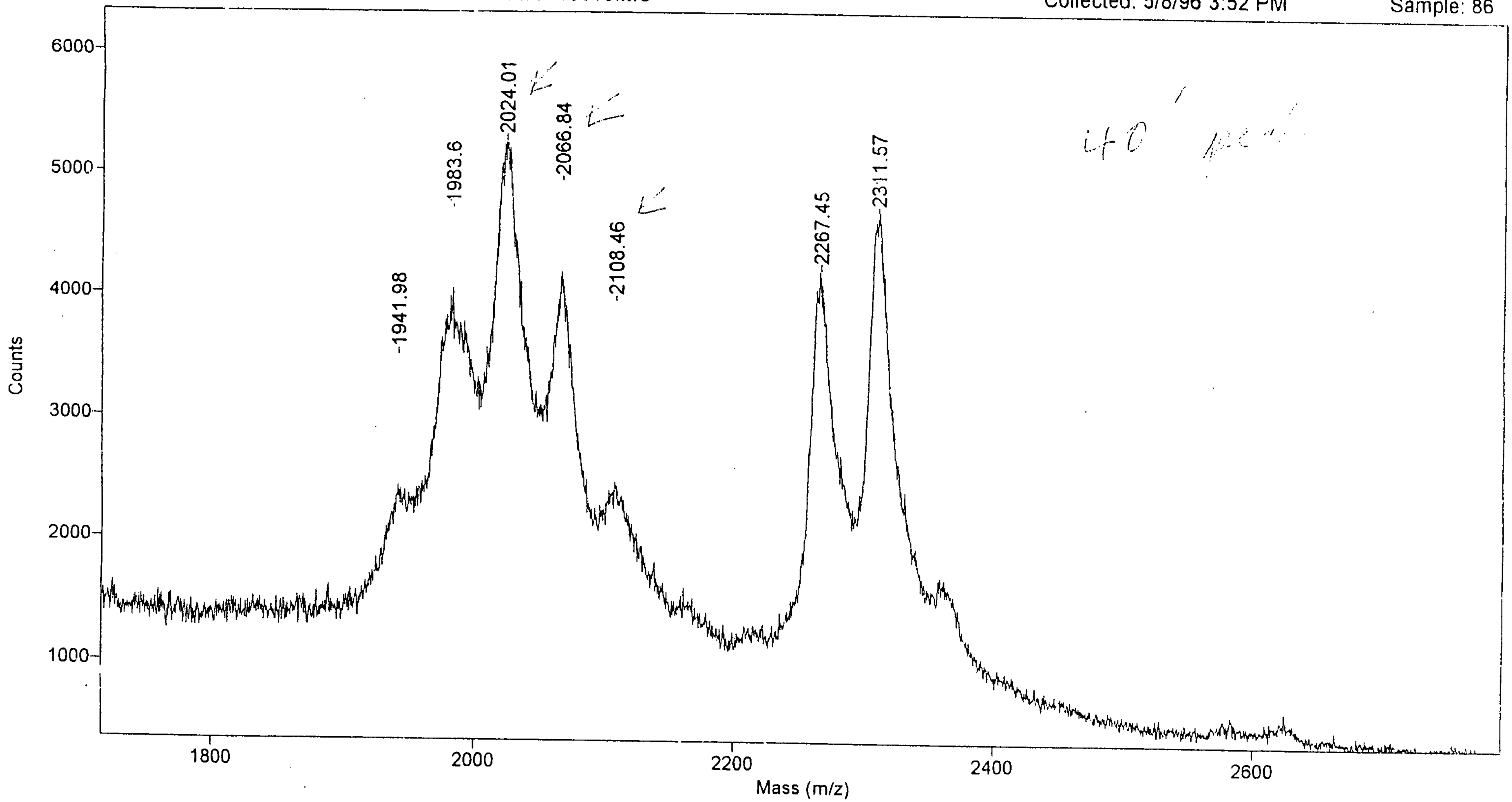
Boston Biomedical Research Institute

Original Filename: c:\voyager\data\vic\96050810.ms

This File # 2 = C:\VOYAGER\DATA\VIC\96050810.MS

Collected: 5/8/96 3:52 PM

Sample: 86



B001470

Comment: Exchanged BP-RLC-C165, from gels, slow cross-linked product, after HPLC, 56-60 min

Method: PEPL
Mode: Linear

Accelerating Voltage: 30000
Grid Voltage: 70.0 %
Guide Wire Voltage: 0.100 %
Negative Ions: OFF

Laser : 400
Scans Averaged: 256
Pressure: 1.31e-06

Low Mass Gate: OFF
Timed Ion Selector: 1364.3

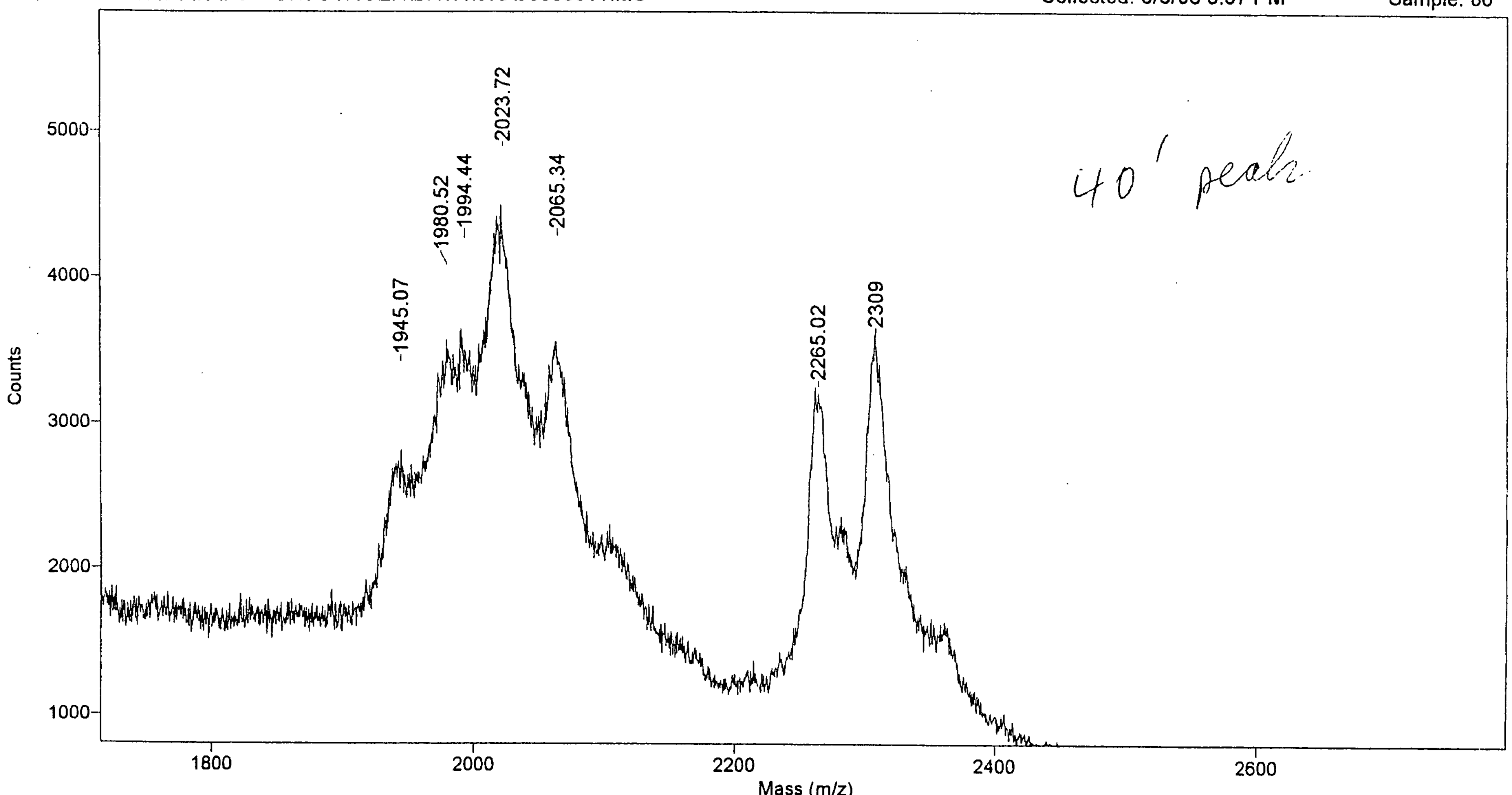
Mirror Ratio: 1.050
PSD Mirror Ratio:

Acquisiti[illegible] Cal File: C:\VOYAGER\DATA\VIC\PEPL.USR

Boston Biomedical Research Institute

Original Filename: c:\voyager\data\vic\96050811.ms

This File # 3 = C:\VOYAGER\DATA\VIC\96050811.MS   Collected: 5/8/96 3:57 PM   Sample: 86



B001471

Comment: Exchanged BP-RLC-C165, from gels, slow cross-linked product, after HPLC, 56-60 min

Method: PEPL	Accelerating Voltage: 30000	Laser : 400	Low Mass Gate: OFF	Mirror Ratio: 1.050
Mode: Linear	Grid Voltage: 70.0 %	Scans Averaged: 256	Timed Ion Selector: 1364.3	PSD Mirror Ratio:
	Guide Wire Voltage: 0.100 %	Pressure: 4.96e-07		
	Negative Ions: OFF	Acquisition Cal File: C:\VOYAGER\DATA\VIC\PEPL.USR		

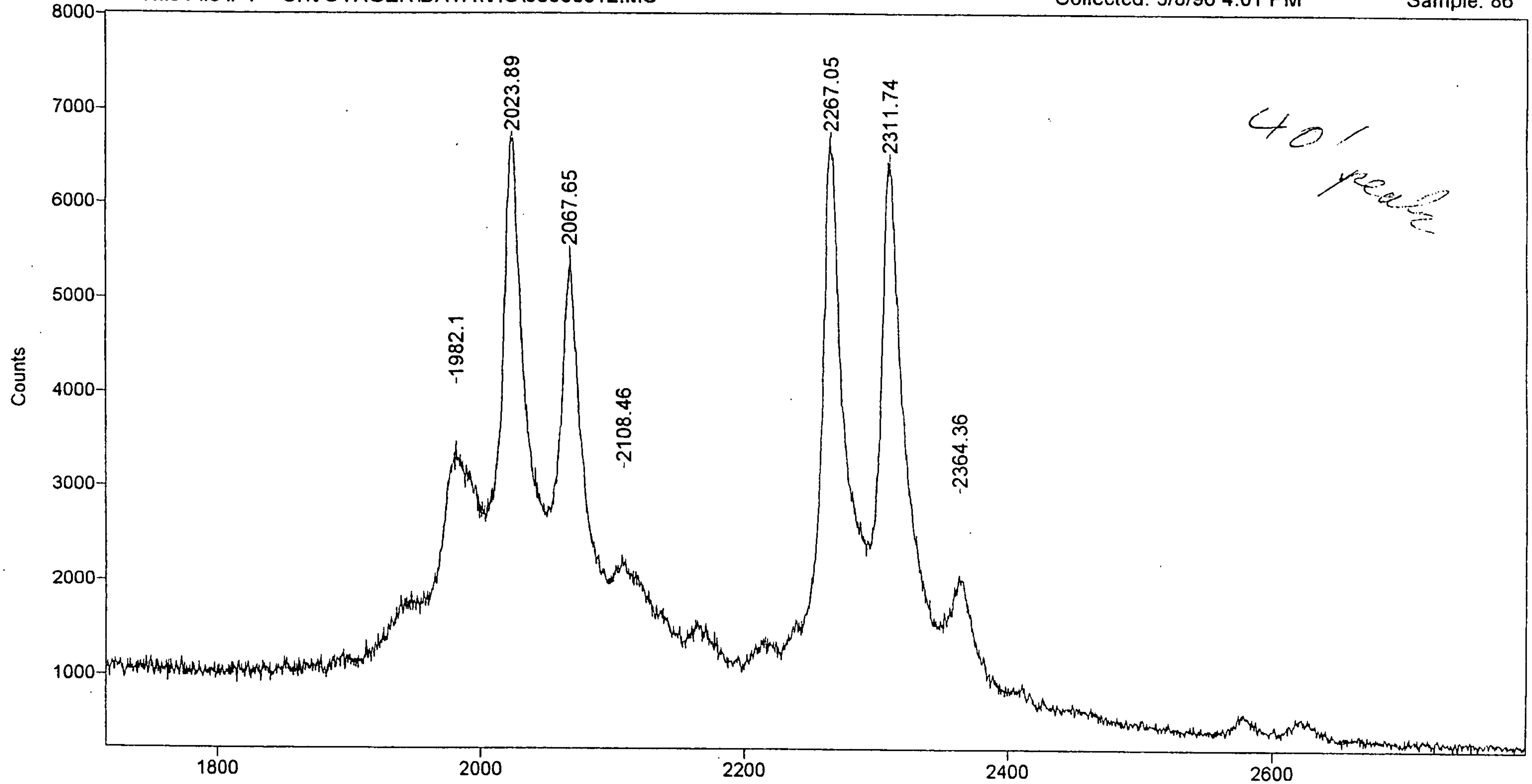
Boston Biomedical Research Institute

Original Filename: c:\voyager\data\vic\96050812.ms

This File # 4 = C:\VOYAGER\DATA\VIC\96050812.MS

Collected: 5/8/96 4:01 PM

Sample: 86



B001472

Comment: Exchanged BP-RLC-C165, from gels, slow cross-linked product, after HPLC, 56-60 min

Method: PEPL
Mode: Linear

Accelerating Voltage: 30000
Grid Voltage: 70.0 %
Guide Wire Voltage: 0.100 %
Negative Ions: OFF

Laser : 400
Scans Averaged: 256
Pressure: 3.01e-07
Acquisition Cal File: C:\VOYAGER\DATA\VIC\PEPL.USR

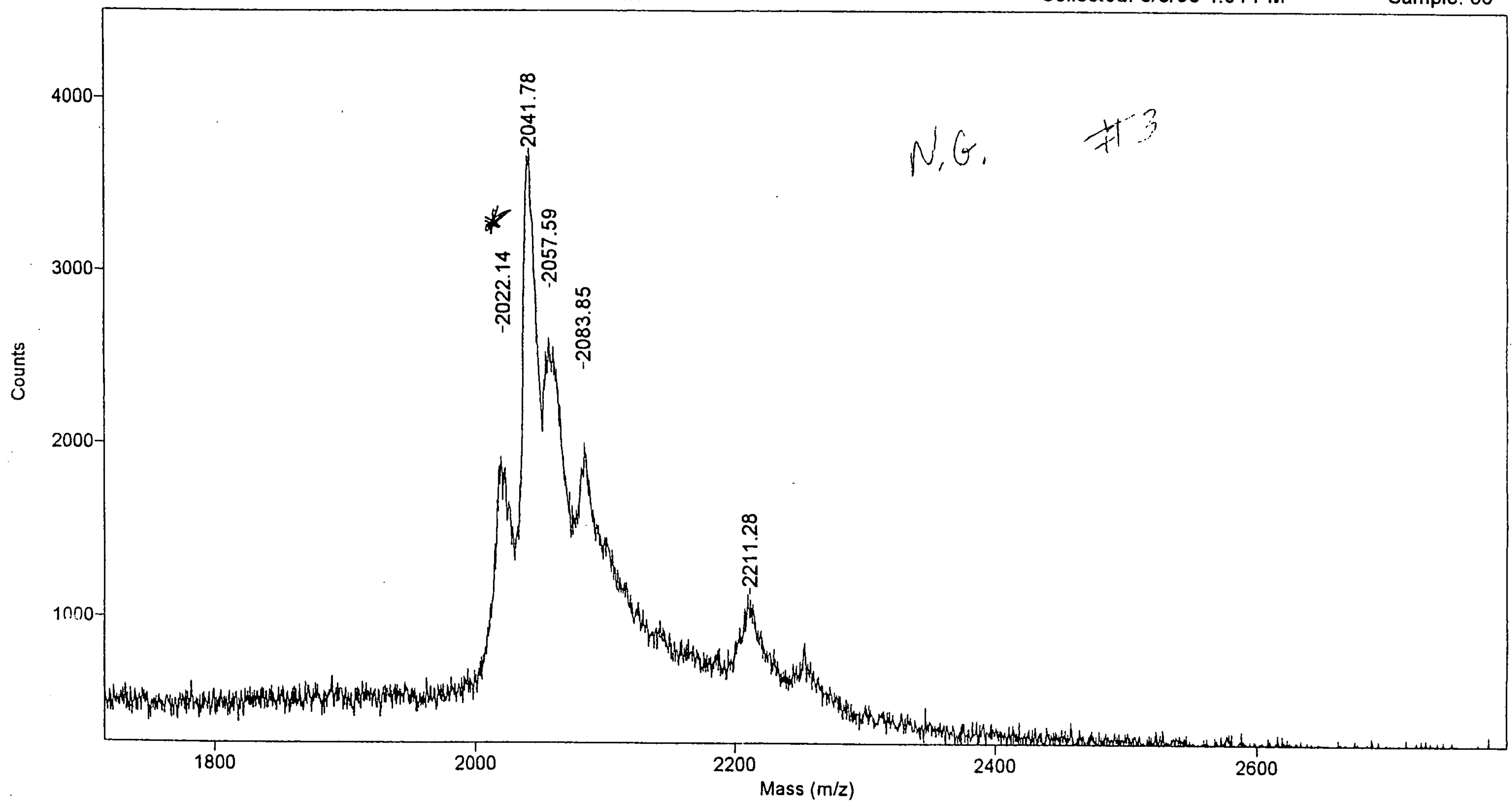
Low Mass Gate: OFF
Timed Ion Selector: 1364.3

Mirror Ratio: 1.050
PSD Mirror Ratio:

Boston Biomedical Research Institute

Original Filename: c:\voyager\data\vic\96050813.ms

This File # 5 = C:\VOYAGER\DATA\VIC\96050813.MS | Collected: 5/8/96 4:04 PM | Sample: 86



Comment: Exchanged BP-RLC-C165, from gels, slow cross-linked product, after HPLC, 56-60 min

B001473

Method: PEPL	Accelerating Voltage: 30000	Laser : 400	Low Mass Gate: OFF	Mirror Ratio: 1.050
Mode: Linear	Grid Voltage: 70.0 %	Scans Averaged: 256	Timed Ion Selector: 1364.3	PSD Mirror Ratio:
	Guide Wire Voltage: 0.100 %	Pressure: 2.25e-07		
	Negative Ions: OFF	Acquisition Cal File: C:\VOYAGER\DATA\VIC\PEPL.USR		

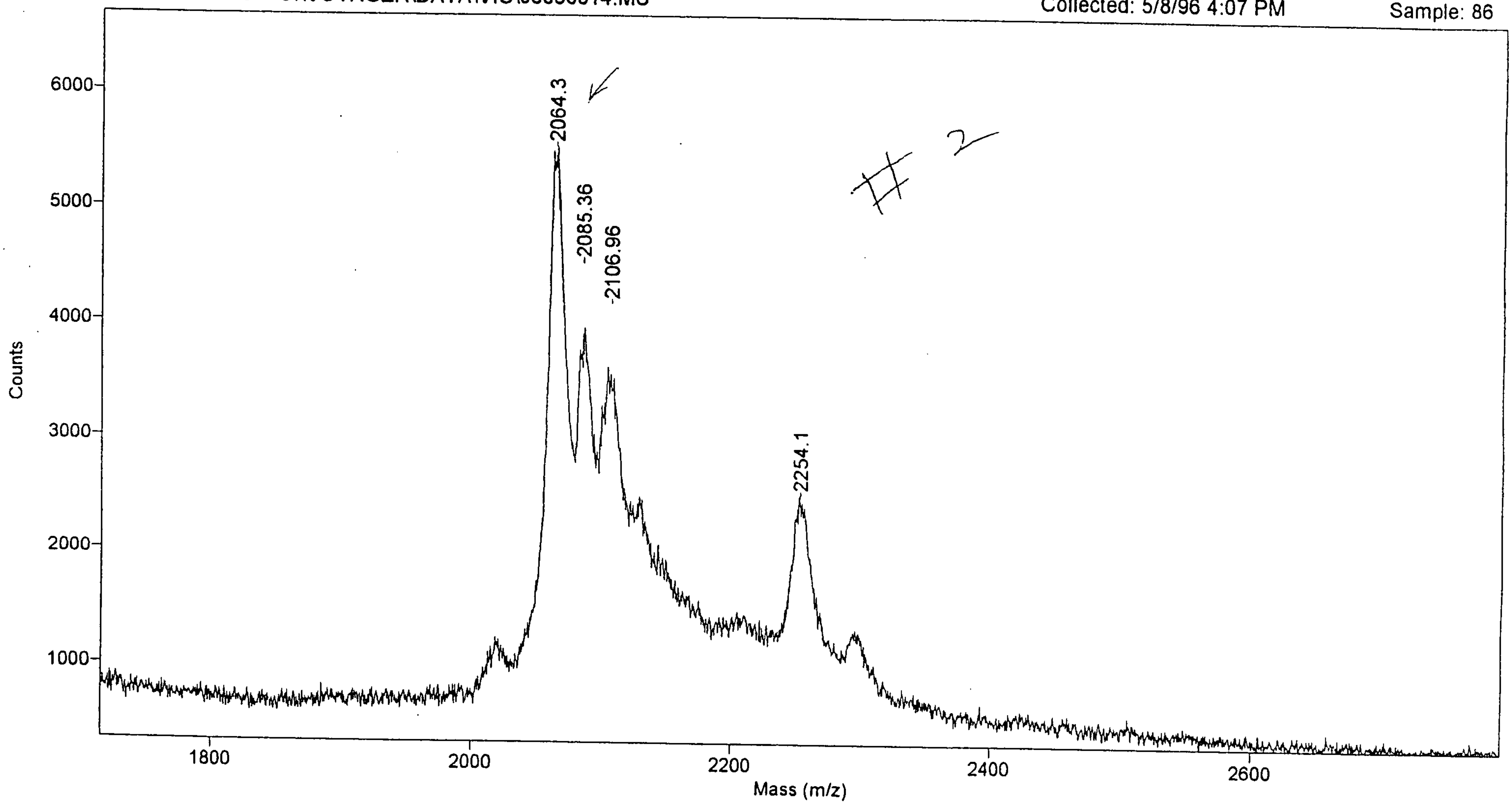
Boston Biomedical Research Institute

Original Filename: c:\voyager\data\vic\96050814.ms

This File # 6 = C:\VOYAGER\DATA\VIC\96050814.MS

Collected: 5/8/96 4:07 PM

Sample: 86



Comment: Exchanged BP-RLC-C165, from gels, slow cross-linked product, after HPLC, 56-60 min

B001474

Method: PEPL

Mode: Linear

Accelerating Voltage: 30000

Grid Voltage: 70.0 %

Guide Wire Voltage: 0.100 %

Negative Ions: OFF

Laser : 400

Scans Averaged: 256

Pressure: 1.92e-07

Acquisition Cal File: C:\VOYAGER\DATA\VIC\PEPL.USR

Low Mass Gate: OFF

Timed Ion Selector: 1364.3

Mirror Ratio: 1.050

PSD Mirror Ratio:

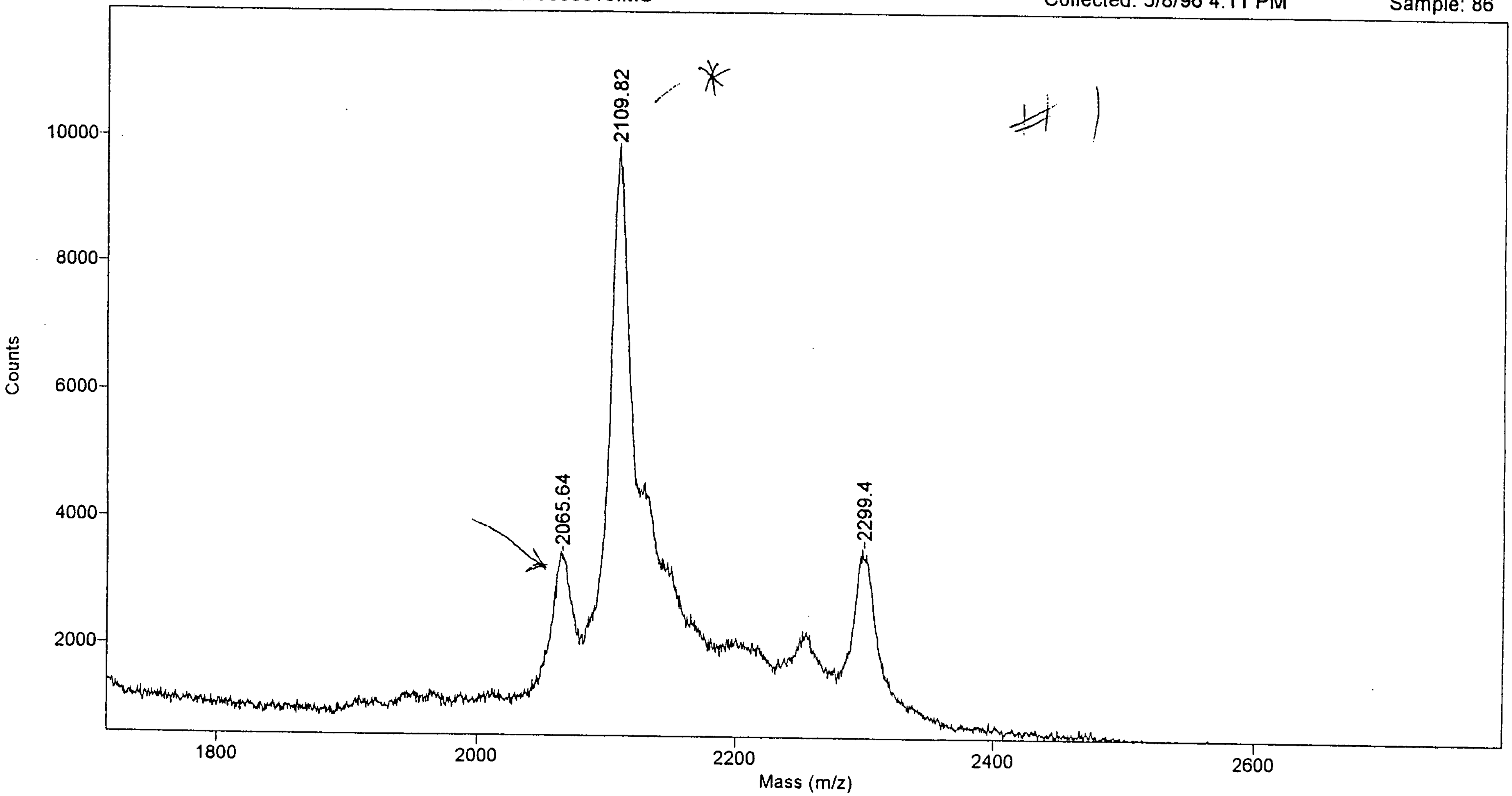
Boston Biomedical Research Institute

Original Filename: c:\voyager\data\vic\96050815.ms

This File # 7 = C:\VOYAGER\DATA\VIC\96050815.MS

Collected: 5/8/96 4:11 PM

Sample: 86



Comment: Exchanged BP-RLC-C165, from gels, slow cross-linked product, after HPLC, 56-60 min

B001475

Method: PEPL

Mode: Linear

Accelerating Voltage: 30000

Grid Voltage: 70.0 %

Guide Wire Voltage: 0.100 %

Negative Ions: OFF

Laser : 400

Scans Averaged: 256

Pressure: 1.55e-07

Acquisition Cal File: C:\VOYAGER\DATA\VIC\PEPL.USR

Low Mass Gate: OFF

Timed Ion Selector: 1364.3

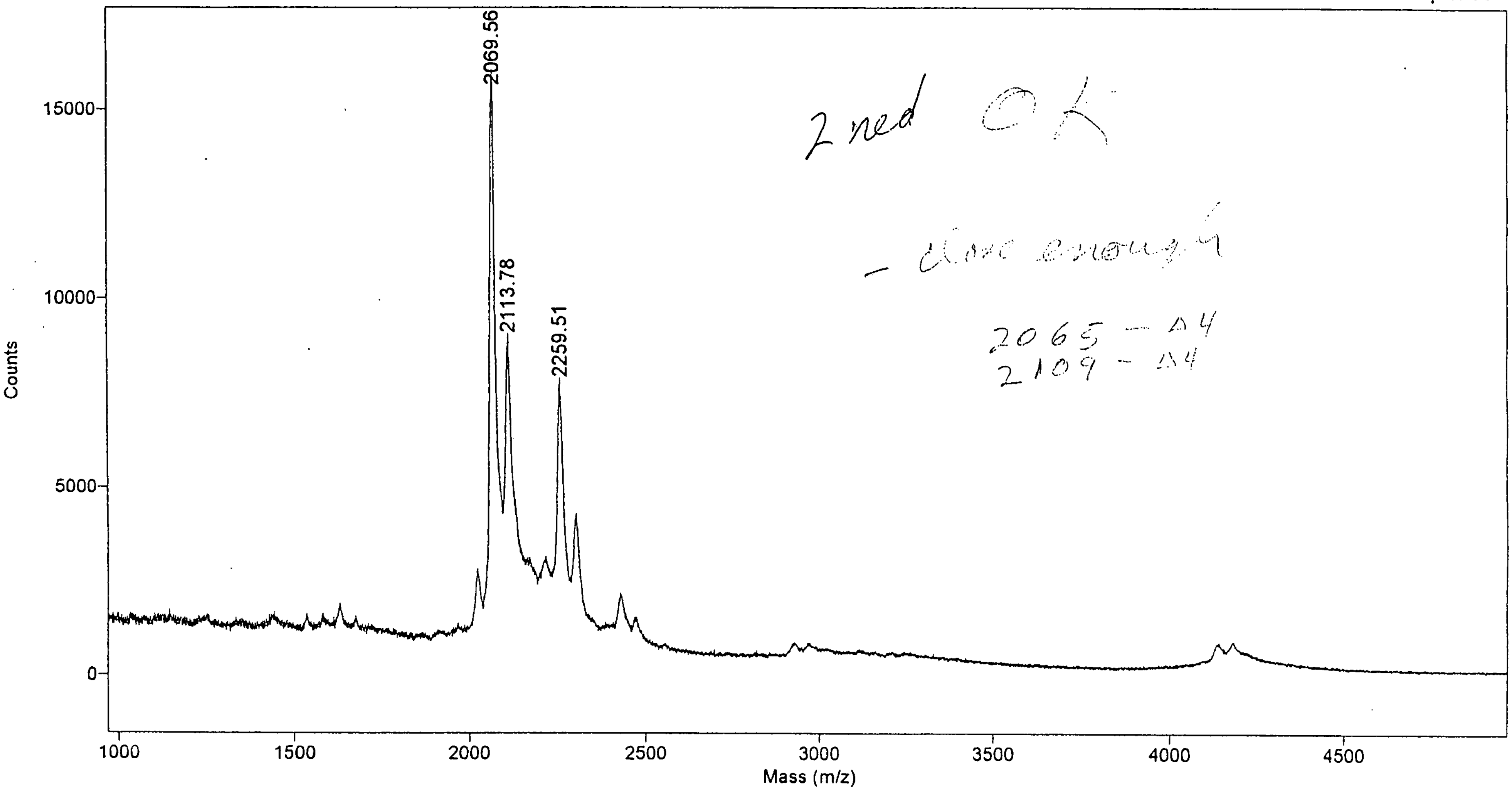
Mirror Ratio: 1.050

PSD Mirror Ratio:

Boston Biomedical Research Institute

Original Filename: c:\voyager\data\vic\96050914.ms

This File # 5 = C:\VOYAGER\DATA\VIC\96050914.MS | Collected: 5/10/96 10:40 AM | Sample: 95



Comment: Exchanged BP-RLC-Cys5, from gels, u/l cross-linked band, tyrpsin O/V, after HPLC, min 71-74

B001476

Method: PEPL
Mode: Linear

Accelerating Voltage: 30000
Grid Voltage: 70.0 %
Guide Wire Voltage: 0.100 %
Negative Ions: OFF

Laser : 400
Scans Averaged: 256
Pressure: 1.20e-06
Acquisition Cal File: C:\VOYAGER\DATA\VIC\PEPL.USR

Low Mass Gate: OFF
Timed Ion Selector: 1364.3

Mirror Ratio: 1.050
PSD Mirror Ratio:

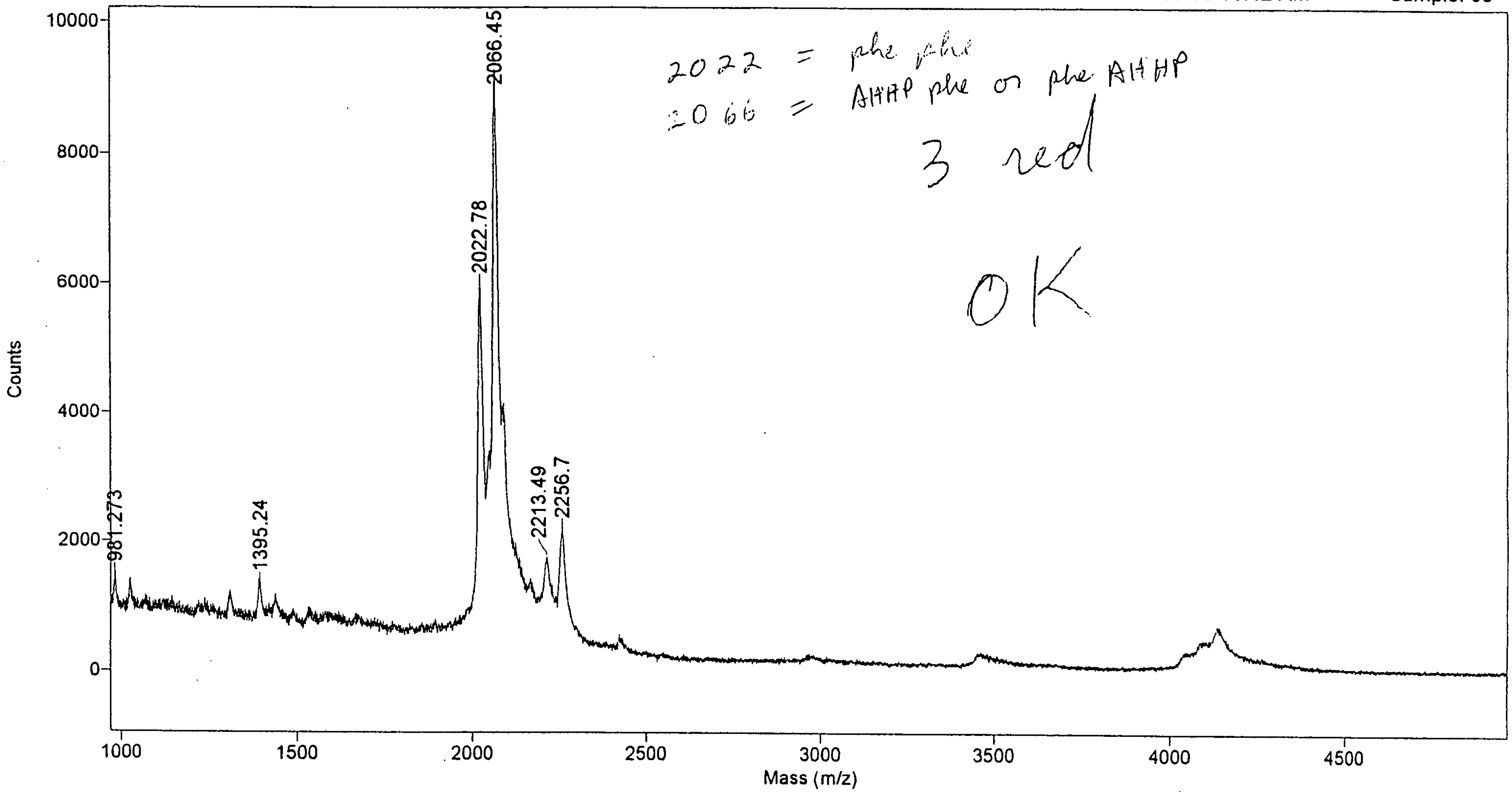
Boston Biomedical Research Institute

Original Filename: c:\voyager\data\vic\96050915.ms

This File # 6 = C:\VOYAGER\DATA\VIC\96050915.MS

Collected: 5/10/96 10:42 AM

Sample: 95



Comment: Exchanged BP-RLC-Cys5, from gels, u/l cross-linked band, tyrpsin O/V, after HPLC, min 71-74

B001477

Method: PEPL
Mode: Linear

Accelerating Voltage: 30000
Grid Voltage: 70.0 %
Guide Wire Voltage: 0.100 %
Negative Ions: OFF

Laser : 400
Scans Averaged: 256
Pressure: 6.83e-07
Acquisition Cal File: C:\VOYAGER\DATA\VIC\PEPL.USR

Low Mass Gate: OFF
Timed Ion Selector: 1364.3

Mirror Ratio: 1.050
PSD Mirror Ratio:

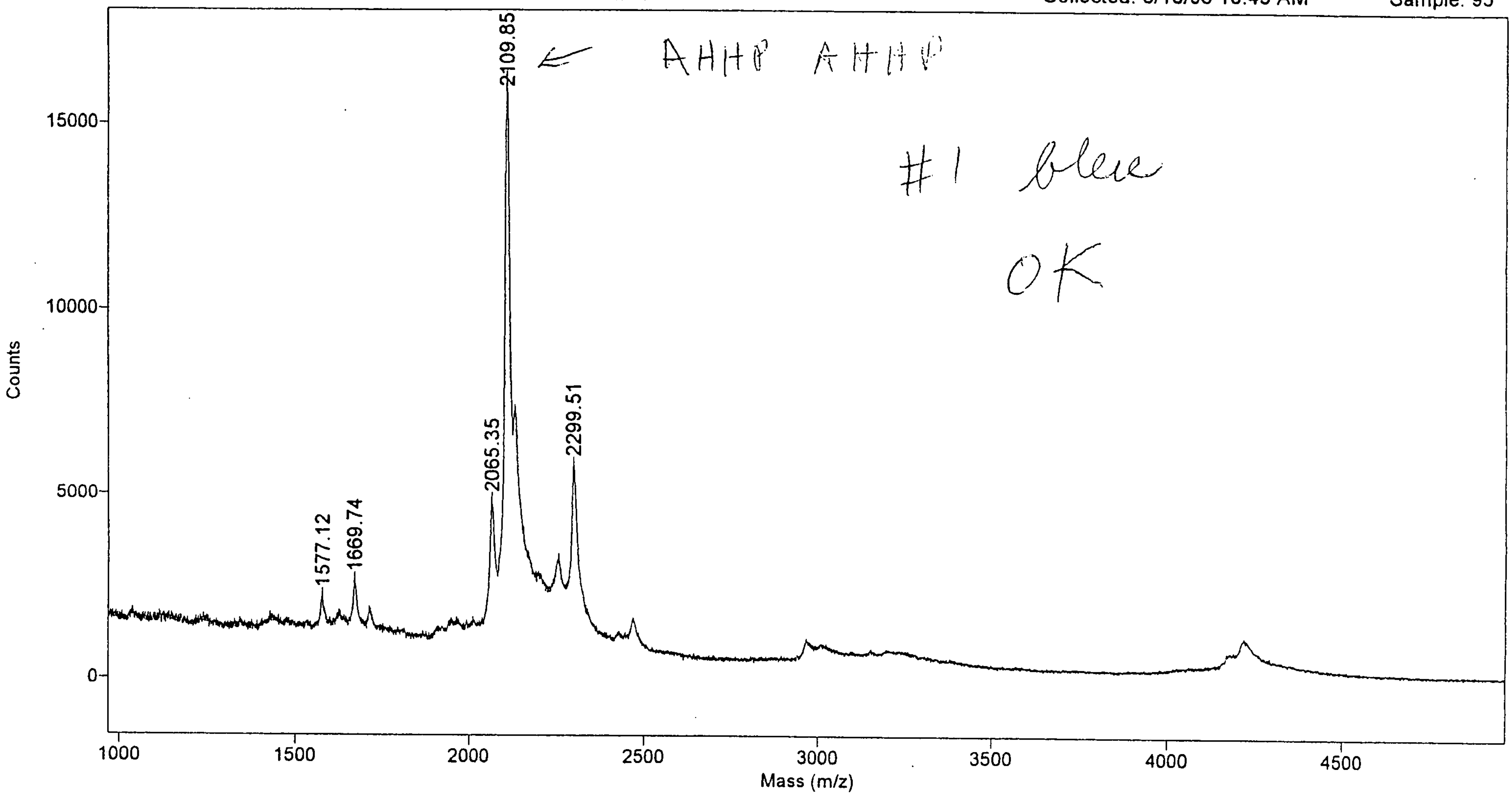
Boston Biomedical Research Institute

Original Filename: c:\voyager\data\vic\96050916.ms

This File # 7 = C:\VOYAGER\DATA\VIC\96050916.MS

Collected: 5/10/96 10:45 AM

Sample: 95



Comment: Exchanged BP-RLC-Cys5, from gels, u/l cross-linked band, tyrpsin O/V, after HPLC, min 71-74

B001478

Method: PEPL

Mode: Linear

Accelerating Voltage: 30000

Grid Voltage: 70.0 %

Guide Wire Voltage: 0.100 %

Negative Ions: OFF

Laser : 400

Scans Averaged: 256

Pressure: 4.39e-07

Acquisition Cal File: C:\VOYAGER\DATA\VIC\PEPL.USR

Low Mass Gate: OFF

Timed Ion Selector: 1364.3

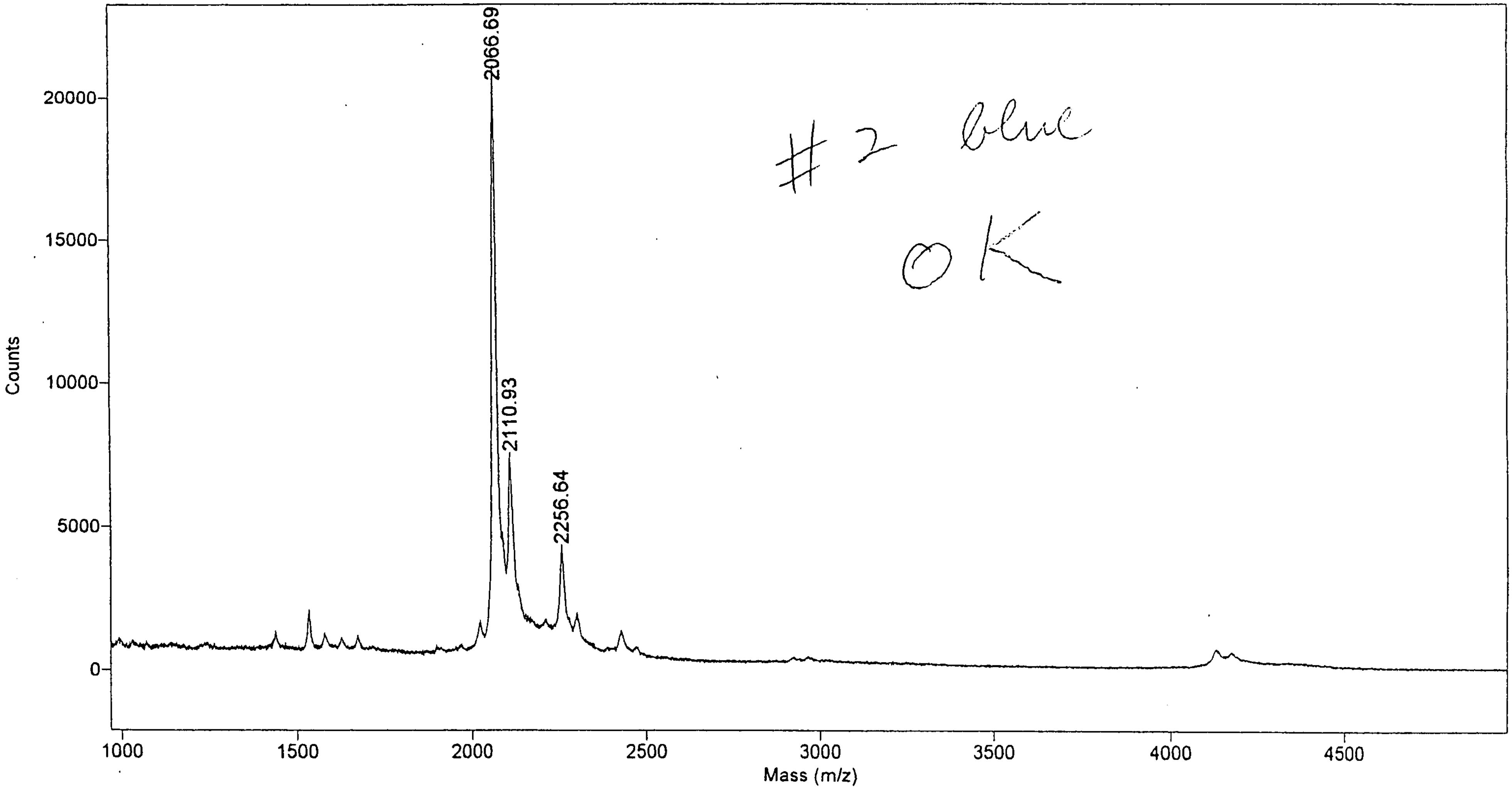
Mirror Ratio: 1.050

PSD Mirror Ratio:

Boston Biomedical Research Institute

Original Filename: c:\voyager\data\vic\96050917.ms

This File # 8 = C:\VOYAGER\DATA\VIC\96050917.MS   Collected: 5/10/96 10:48 AM   Sample: 95



Comment: Exchanged BP-RLC-Cys5, from gels, u/l cross-linked band, tyrpsin O/V, after HPLC, min 71-74   B001479

Method: PEPL   Accelerating Voltage: 30000   Laser : 400   Low Mass Gate: OFF   Mirror Ratio: 1.050

Mode: Linear   Grid Voltage: 70.0 %   Scans Averaged: 256   Timed Ion Selector: 1364.3   PSD Mirror Ratio:

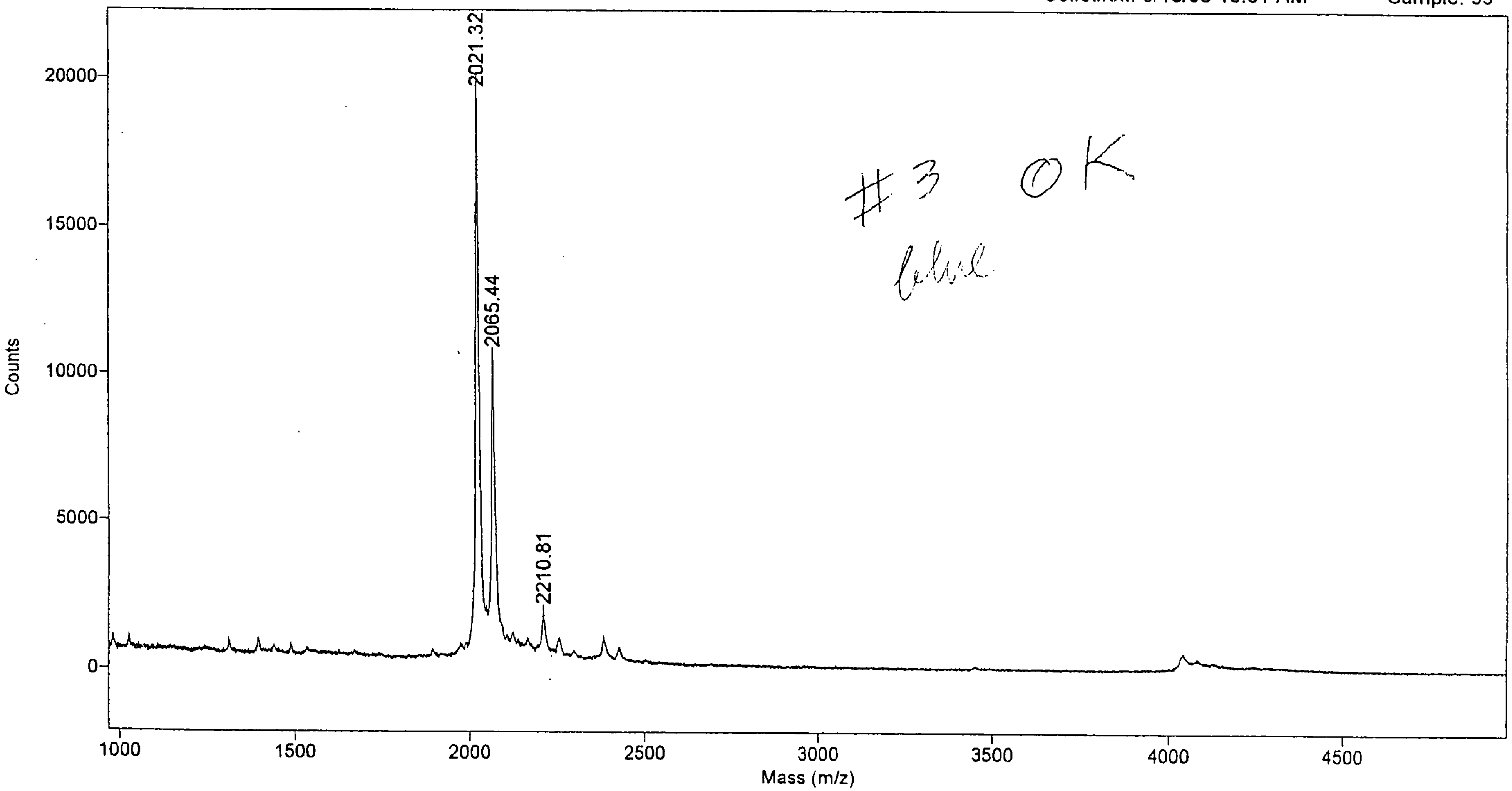
Guide Wire Voltage: 0.100 %   Pressure: 3.30e-07

Negative Ions: OFF   Acquisition Cal File: C:\VOYAGER\DATA\VIC\PEPL.USR

Boston Biomedical Research Institute

Original Filename: c:\voyager\data\vic\96050918.ms

This File # 9 = C:\VOYAGER\DATA\VIC\96050918.MS   Collected: 5/10/96 10:51 AM   Sample: 95



#3 OK

Comment: Exchanged BP-RLC-Cys5, from gels, u/l cross-linked band, tyrpsin O/V, after HPLC, min 71-74   B001480

Method: PEPL
Mode: Linear

Accelerating Voltage: 30000
Grid Voltage: 70.0 %
Guide Wire Voltage: 0.100 %
Negative Ions: OFF

Laser : 400
Scans Averaged: 256
Pressure: 2.74e-07
Acquisition Cal File: C:\VOYAGER\DATA\VIC\PEPL.USR

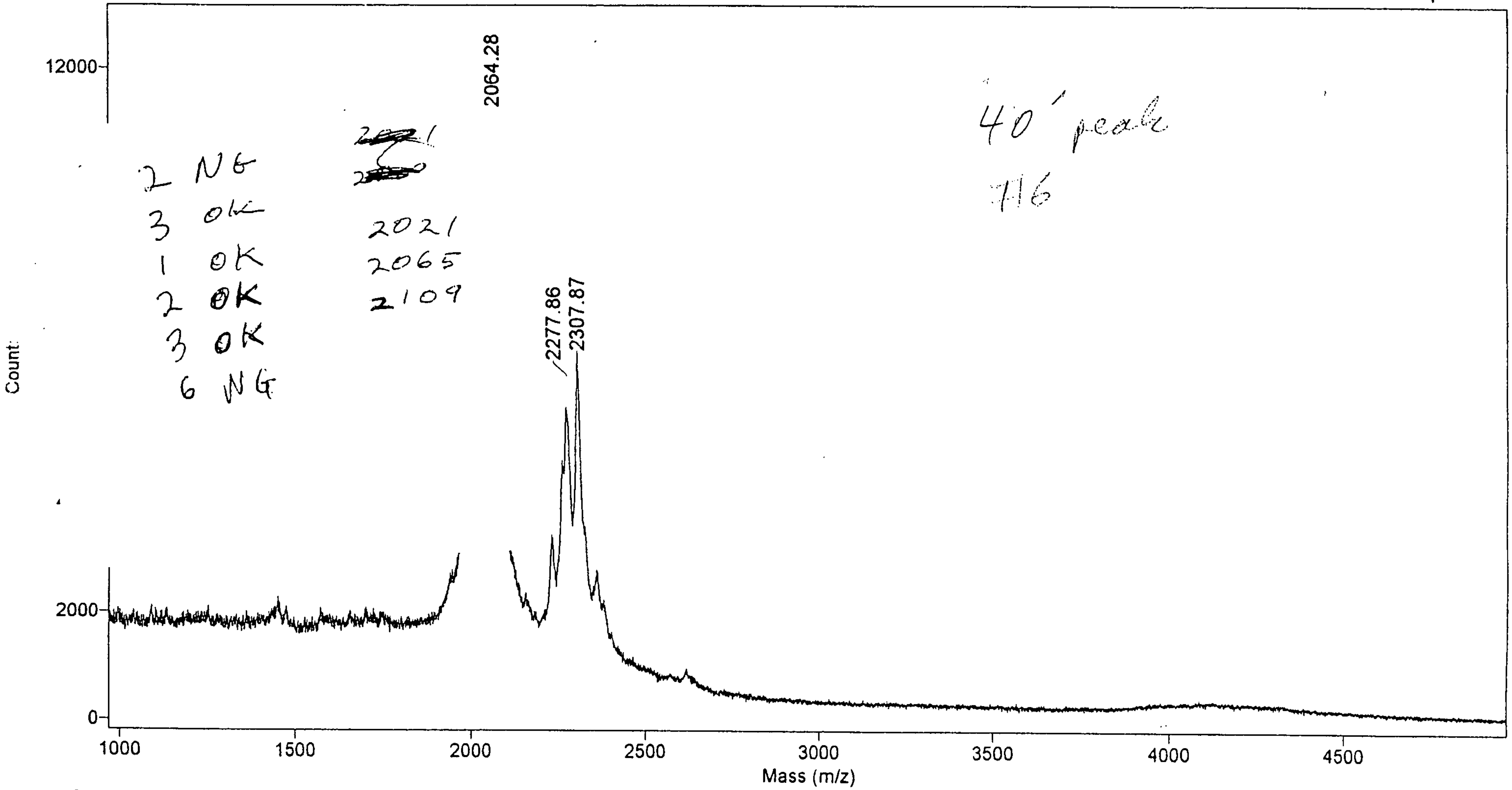
Low Mass Gate: OFF
Timed Ion Selector: 1364.3

Mirror Ratio: 1.050
PSD Mirror Ratio:

Boston Biomedical Research Institute

Original Filename: c:\voyager\data\vic\96050920.ms

This File #11 = C:\VOYAGER\DATA\VIC\96050920.MS   Collected: 5/10/96 10:58 AM   Sample: 95



Comment: Exchanged BP-RLC-Cys5, from gels, u/l cross-linked band, tyrpsin O/V, after HPLC, min 71-74   B001481

Method: PEPL
Mode: Linear

Accelerating Voltage: 30000
Grid Voltage: 70.0 %
Guide Wire Voltage: 0.100 %
Negative Ions: OFF

Laser : 500
Scans Averaged: 256
Pressure: 1.99e-07
Acquisition Cal File: C:\VOYAGER\DATA\VIC\PEPL.USR

Low Mass Gate: OFF
Timed Ion Selector: 1364.3

Mirror Ratio: 1.050
PSD Mirror Ratio:

	A	B	C	D
1	B 370 (1)	50ug i.p. in CFA 7/25/94	40ug ip in IFA 4/28/95	
2	Bser (2)	25ug in CFA 4/25/95		
3	A190 in IFA (1)	50ug.mL in CFA 5/10/95	boost 40ug/mL in IFA	
4	PS II + RIBI (3)	see protocol 1/22/96	see protocol 2/6/96	
5	PO4 - TS- KLH (1)	200ul in CFA 4/12/96	200ul in IFA 5/6/96	
6	Alz-TS-KLH (4)	50 ug in CFA 5/14/96	50 ug in IFA 8/7/96	
7	FUS-KLH (3)	50 ug in CFA 6/28/96	50 ug in IFA 8/7/96	
8				
9				
10				
11	have 1 clean cage with 5 mice	Also have 2 mice in fusin cag	e that have not been touched	
12				
13				
14				
15				
16				
17				
18				
19				
20				

B001482

Christine's Notes

FUSION 8/30/96

spleen cells

$130 \div 4 \times 20 = 6.5 \times 10^2 \times 10^4$

$= 6.5 \times 10^6 \times 15 mL$

$= 9.7 \times 10^7$ cells

NS-1 cells

$2 \times 10^6/mL \times 50 mL = 1 \times 10^8$ cells

8 plates

160 mL

HAT 160/100 = 1.6 mL

origen 10% = 16 mL

* Made new Hy, sterile PBS & used new lot of PEG & 10% origen

Fusion Procedure

- Used a mouse previously immunized with a PO_4 transition state + KLH
- Immunized on 4/12/96 in CFA + on 5/16/96 in IFA
- IP the final boost was done on 8/8/96 IV
- Used NS-1 cells in fusion

- Cell Counts:

Spleen 28 ÷ 4 = 7 x 20 1.4×10^7 spleen

NS-1 90 ÷ 4 = 22.5 x 20 4.5×10^7 NS-1

1:4 ratio

- Brought up cells in 160 ml's of Hy medium containing 1 X HAT, used 8 plates, 200 uL/well

Fusion Done on 10/22/96

spleen cells

$190/4 \times 20 \times 10^4 = 9.5 \times 10^6/mL = 9.5 \times 10^7$ cells

NS-1 cells

$114/4 \times 20 \times 10^4 = 5.7 \times 10^6/mL = 5.7 \times 10^7$ cells

9/6/96

Fusion

- Sacrificed Immunized mouse and removed spleen
The spleen was much larger than normal
- Spleen was teased apart in serum free med. and pipetted into a 15 mL tube
Clumps settled in tube and then poured cells into a clean 15 mL tube avoiding the large clumps getting in.
- Spun down spleen cells as well as NS-1 cells
- Counted spleen & NS-1 cells, & used a 1:1 ratio Combined cells together & spun down in H for 10 min.
- Discard the medium completely so you are left with a dry pellet DO NOT DISRUPT PELLET use a pasteur pipet to get any excess medium. Put in H_2O bath
- Add 1 mL PEG (we used a new lot) & place in H_2O Bath for 1 min
- Dilute PEG by adding serum free medium
  1 mL SFM → 1 min H_2O bath
  2 mL " → 2 " "
  4 mL " → 4 " "
  8 mL " → spin tube for 10 min.
- Discard medium and bring up to desired volume in Hy (made up fresh w/ new OPI) & filtered in washed .45 filter
Hy contains HAT + new cloning supplement.
- We plate 1/2 plates with cells that were in medium containing the cloning supplement + 1/2 that only had Hy + HAT

B001487

Calculations

Spleen

because it's a 1:20 dilution

$147/4 \times 20 \times 10^4 = 7.35 \times 10^6$ cells/ml
$\times 10$ mL
7.35×10^7 cells

NS-1

$92/4 \times 20 \times 10^4 = 4.6 \times 10^6$ cells/ml
$\times 10$ mL
4.6×10^7 cells

Fusion 9/13/96

Spleen

$135/4 \times 20 \times 10^4 = 6.75 \times 10^6$

$\times$ 10mL

6.75×10^7 cells

NS-1

$211/4 \times 20 \times 10^4 = 1.05 \times 10^7$

$\times$ 10mL

1.05×10^8 cells

In this fusion we used the same procedure as for the 9/6/96 fusion. This time we used cloning supplement in <u>all</u> plates. We noticed on the 9/6/96 fusion that the plates w/out C.S. were growing better than the ones w/ C.S. On 9/16/96 we

tried to rescue the cells
by removing the medium
that was in the wells
+ adding thy w/ just
HAT supplement, no C.S.

DT → PCR 481 Eco Nde } purified ligated together

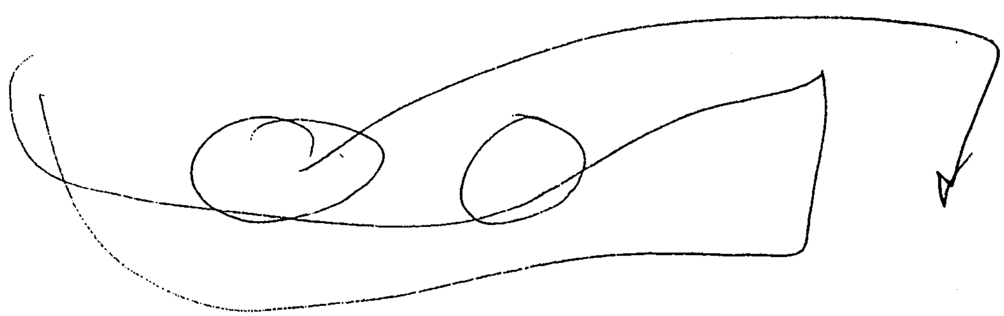
Vector → grew up

EcoRI NdeI, carb.

Eco Nde

Comp cells

E coli → E coli 481



481

B001491

Injecting Mice for Production of Ascites

- After cell line has grown up in culture flask transfer into a 15 mL tube
- Add more HY medium to the culture flask and return to incubator
- Spin down the 15 mL tube (on 4 for 10 min)
- Discard medium & resuspend in .5 mL of sterile filtered PBS.
- Draw up cells into insulin syringe (the 15 mL tube may need to be cut in half in order for the syringe to fit)
- Air bubbles in the syringe for an IP injection is not as important as an IV injection
- Inject cell line into corresponding mice (IP)
- Mark on card what cell line corresponds to which mouse. Do this by the ear hole punch:

  Rt
  Lt
  Both
  2 Rt

Fusions

PS II
w/ ~~[illegible]~~ heterologous boost

ALZ
Changes in the 2 phenylalanines

B001493

SynthAssist™ Peptide Synthesis

Report type: Sequence
10/24/96 @ 1:39 PM
File name: Raso 10mer Statine Pep Se(

Sequence editor:

Sequence : Cys-Met-Val-Gly-Gly-Val/Sta-Val/Sta-Ile/Sta-Ala-Thr

Comments : V. Raso 10mer 0.25 mmol scale. Cap with 0.45gm benzoic Anhydride
HMP resin=0.89mmol/gm subst=0.281gm. N-Terminal Deprotect.
V6&V7= 0.5mmol each of Fmoc-Val and Fmoc-Sta (200mg)
Ile8=0.5mmol each of Ile-Fmoc and Fmoc-Sta(200mg)

Disulfide Bonds: 0
Chemistry : Fmoc

solid support then add HF to cleave off peptide

Composition:

Sequence : [H]-Cys-Met-Val-Gly-Gly-Val/Sta-Val/Sta-Ile/Sta-Ala-Thr-[OH]

Composition : C40 H72 N10 O12 S2
Weight : 949.2016
C-Terminal : OH
N-Terminal : H
# of Residues : 10
AA List :

AA	Count
Ala	1
Cys	1
Gly	2
Ile	1
Met	1
Thr	1
Val	3

949.2
58.1
58.1
44.0
1109.4

949.2
58.1
44.0
1051.3

949.2
58.1
+ 58.1
1065.4

949.2
44.0
993.2 I.L

949.2
+ 58.1 Val
1007.3

Alzheimers peptide

Calculations:
(Calculation not updated)

Sta Δ

MW Val = 99.1
MW Ile = 113.2
MW Sta = 157.2
X = Sta

8 different peptides

	Val/Sta	Val/Sta	Ile/Sta
	X	O	O
made 1007.3 →	O	X	O
993.2 →	O	O	X
1065.4 →	X	O	O
1051.3 →	O	X	X
	X	O	X
949.2 →	O	O	O
MW 1109.4 →	X	X	X

113.2
+ 44
157.2 Sta
− 99.1 Val
58.1 Δ

157.2 St
113.2 Ile
44.0

949.2 ← MW 949.2

B001494

Boston Biomedical Research Institute

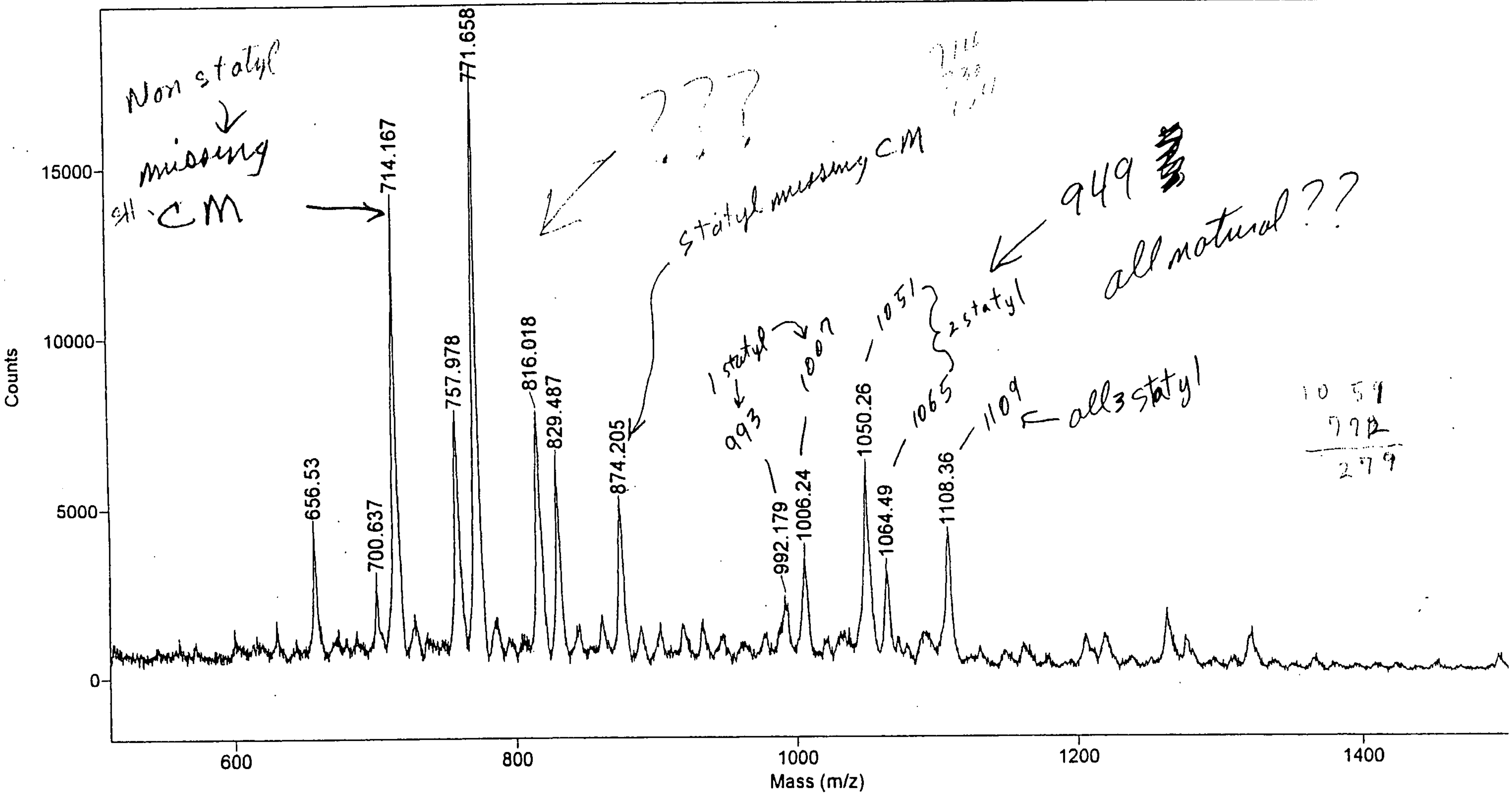
Original Filename: c:\voyager\data\vic\61030076.ms

This File # 3 = C:\VOYAGER\DATA\VIC\61030076.MS

Collected: 11/5/96 1:02 PM

Sample: 10

MASS SPEC



B001495

Comment:

Method: PEPL

Mode: Linear

Accelerating Voltage: 30000

Grid Voltage: 70.0 %

Guide Wire Voltage: 0.100 %

Negative Ions: OFF

Laser : 450

Scans Averaged: 256

Pressure: 8.99e-08

Low Mass Gate: OFF

Mirror Ratio: 1.050

PSD Mirror Ratio:

Timed Ion Selector: 1364.3

Boston Biomedical Research Institute
Peptide Synthesis Requisition Form
ABI 431A Synthesizer

Please complete the following form and submit it to Anna Wong for each peptide to be synthesized.

Date 10/24

Investigator Name: Vic Raso Ext# 316

Department:

Sequence (use 3 letter code):

H_2N- Cys - Met - Val - Gly - Gly - (Val/sta) - (Val/sta) - (Ile/sta) - Ala - Thr - -COOH

The Amino Acids in stock are:

Fmoc-L-Ala	Fmoc-Gln(Trt)	Fmoc-L-Leu	Fmoc-L-Ser(tBu)
Fmoc-L-Arg(Pmc)	Fmoc-L-Glu(OtBu)	Fmoc-L-Lys(Boc)	Fmoc-L-Thr(tBu)
Fmoc-Asn(Trt)	Fmoc-Gly	Fmoc-L-Met	Fmoc-L-Trp
Fmoc-L-Asp(OtBu)	Fmoc-L-His(Trt)	Fmoc-L-Phe	Fmoc-L-Tyr(tBu)
Fmoc-L-Cys(Trt)	Fmoc-L-Ile	Fmoc-L-Pro	Fmoc-L-Val)

Choose a Resin: Rink Resin (Amide) ____ HMP Resin (Acid) X

Choose a Scale: 0.10mmole ____ (for 20 residue, assuming 80-90% cleavage recovery, yields about 150-175 mg crude peptide)

0.25mmole X (for 20 residue, assuming 80-90% cleavage recovery, yields about 390-440 mg crude peptide)

Do you want the N-Terminal Fmoc removed? Yes X No ____

Additional Instructions anna - I think you should remove 1/2 of the contents of the val, val, Ile and add 200 mg of sta to each for the mixture

Operators Comments

Thai Leit OK, Started 10/24/96

Fmoc sta = 1 mmole = 397 mg

0.5 mmole = ~ 200 mg

Fees: Set-Up Charge	$25.00
0.25mmole Scale	$12.00/Amino Acid
0.10mmole Scale	$10.00/Amino Acid

Cleavage of the peptide from the resin is not included in this service!!!

B001496

ABI MODEL 431A VERSION 2.00 Fmoc

SYNTHESIS REPORT

SEQUENCE NAME: V. Ras 0 10 mer 0.25 scale

RESIN: HMP Resin = 0.281 gm

OPERATOR: A. Wong

RUN EDITOR:

Cy: 1 Rpt: 1 M: d

Cy: 2 Rpt: 1 M: hefffghefffg

Cy: 3 Rpt: 9 M: BADEFG

Cy: 12 Rpt: 1 M: bdc

ADD TIME HOLD CYCLE: 0

RESIN SAMPLES: No

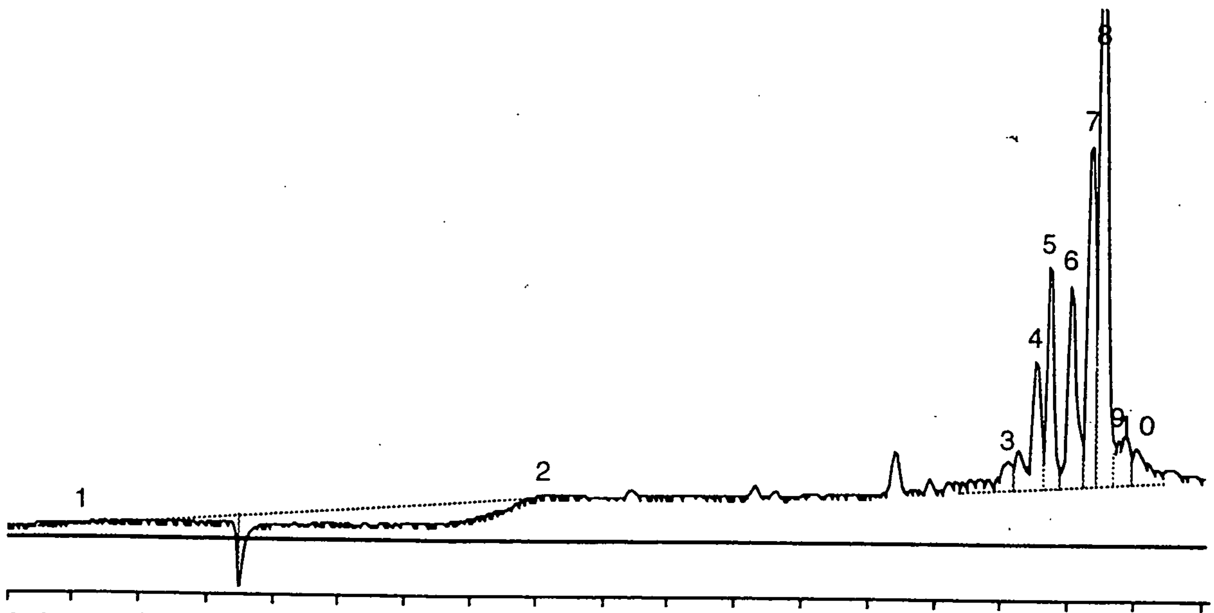
EVENTS LOG:		DATE	TIME	CYCLE	MOD	STEP	SEC REM
Synthesis begun		10/24/96	14:03	1	1	1	1
Barcode read	Thr	10/24/96	14:08	2	1	2	9
Barcode read	Sp1	10/24/96	15:48	2	7	2	9
Barcode read	Ala	10/24/96	17:34	3	2	1	9
Barcode read	~~Sp1~~ ILE/STA	10/24/96	18:28	4	2	1	9
Barcode read	Val/STA	10/24/96	19:22	5	2	1	9
Barcode read	~~Sp1~~ VAL/STA	10/24/96	20:16	6	2	1	9
Barcode read	Gly	10/24/96	21:10	7	2	1	9
Barcode read	Gly	10/24/96	22:05	8	2	1	9
Barcode read	Val	10/24/96	22:59	9	2	1	9
Barcode read	Met	10/24/96	23:53	10	2	1	9

Date: Tue, Oct 29, 1996 3:52 PM
Data: No data saved

Sample: V. Raso ~~5mer~~ 10 mer 25µg injected
Column: C8 analytical 1ml/min
Buffer A: 0.1% TFA; B: 0.1%TFA in 90% CH3CN, 9.9% water
Gradient: 0-60%B, 20'
Monitor: 220nm, 2.0 AUFS; 280nm, 1.0 AUFS

Processing File: profile#1
Method: pepanal 0-60
Sampling Int: 0.1 Seconds

Data:

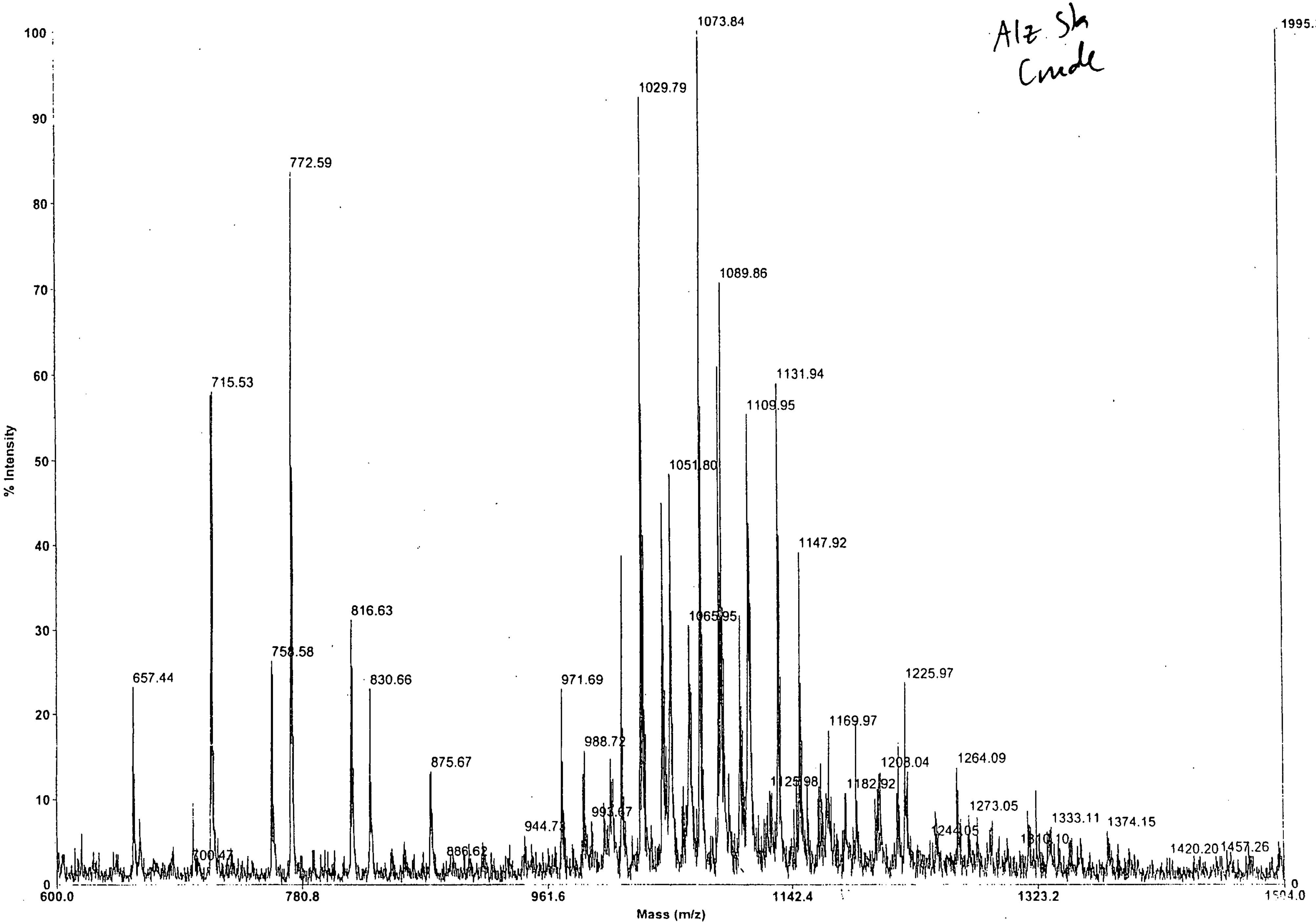


Analysis: Channel A

Peak No.	Time	Type	Height(µV)	Area(µV-sec)	Area%
1	1.156	N1	495	250642	5.122
2	8.123	N2	735	1393453	28.479
3	15.090	N6	7931	147729	3.019
4	15.523	N8	35473	252722	5.165
5	15.728	N9	62018	344400	7.038
6	16.045	N10	56114	420146	8.587
7	16.351	N11	95278	634580	12.969
8	16.506	N12	148970	1131030	23.116
9	16.771	N13	12512	170805	3.490
10	17.040	N14	10119	147254	3.009

B001499

Voyager Spec #1[BP = 243.0, 19050]

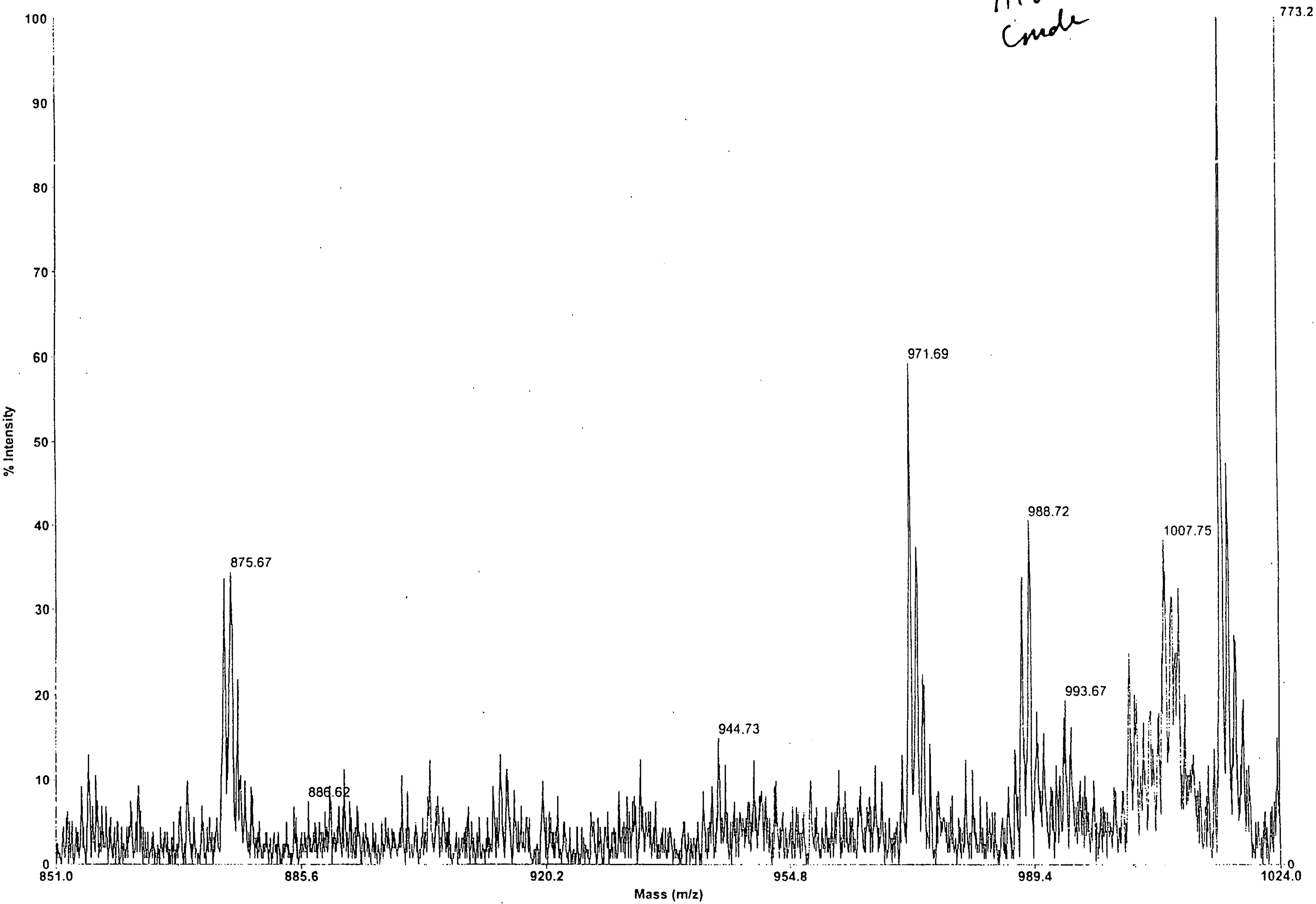


Alz Sta
D:\...\021216_0009.dat
Acquired: 14:44:00, December 16, 2002

B001500

Voyager Spec #1[BP = 243.0, 19050]

Alz sta
Crude



Alz Sta
D:\...\021216_0009.dat
Acquired: 14:44:00, December 16, 2002

B001501

SynthAssist™ Peptide Synthesis

Report type: Sequence ✓
12/5/96 @ 11:14 AM
File name: Raso 14mer 12/5/96 Seq

Sequence editor:

Sequence : Cys-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Tyr-[NH2]

Comments : V. Raso 14mer 0.25scale
Rink Resin=0.81mmol/gm subst=0.308gm
N-terminal Deprotect

14-25 CY

Disulfide Bonds: 0
Chemistry : Fmoc

Composition:

Sequence : [H]-Cys-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Tyr-[NH2]

Composition : C77 H111 N19 O20 S1
Weight : 1654.9171
C-Terminal : NH2
N-Terminal : H
\# of Residues : 14
AA List :

AA	Count
Ala	1
Asp	1
Cys	1
Gln	1
Glu	1
Gly	1
His	1
Leu	1
Lys	1
Phe	2
Tyr	1
Val	2

ALZ
for iodination to test for ~~[illegible]~~ catalytic ALZ PS clones

Calculations:
(Calculation not updated)

O
||
C-NH2

Boston Biomedical Research Institute
Peptide Synthesis Requisition Form
ABI 431A. Synthesizer

14mer

Please complete the following form and submit it to Anna Wong for each peptide to be synthesized.

Date 12/3/96

Investigator Name: Vic Roso Ext# 316

Department:

Sequence (use 3 letter code):

H_2N- Cys - His - Gln - Lys - Leu - Val - Phe - Phe - Ala - Glu - Asp - Val - Gly - Tyr -COOH

The Amino Acids in stock are:

Fmoc-L-Ala	Fmoc-Gln(Trt)	Fmoc-L-Leu	Fmoc-L-Ser(tBu)
Fmoc-L-Arg(Pmc)	Fmoc-L-Glu(OtBu)	Fmoc-L-Lys(Boc)	Fmoc-L-Thr(tBu)
Fmoc-Asn(Trt)	Fmoc-Gly	Fmoc-L-Met	Fmoc-L-Trp
Fmoc-L-Asp(OtBu)	Fmoc-L-His(Trt)	Fmoc-L-Phe	Fmoc-L-Tyr(tBu)
Fmoc-L-Cys(Trt)	Fmoc-L-Ile	Fmoc-L-Pro	Fmoc-L-Val)

Choose a Resin: Rink Resin (Amide) X HMP Resin (Acid) ____

Choose a Scale: 0.10mmole ____ (for 20 residue, assuming 80-90% cleavage recovery, yields about 150-175 mg crude peptide)

0.25mmole X (for 20 residue, assuming 80-90% cleavage recovery, yields about 390-440 mg crude peptide)

Do you want the N-Terminal Fmoc removed? Yes X No ____

Additional Instructions

Operators Comments Flow test OK, started 12/6/96

Fees: Set-Up Charge $25.00
0.25mmole Scale $12.00/Amino Acid
0.10mmole Scale $10.00/Amino Acid

Cleavage of the peptide from the resin is not included in this service!!!

ABI MODEL 431A VERSION 2.00 Fmoc

SYNTHESIS REPORT

SEQUENCE NAME: V. Raso 14mer 0.25 scale

RESIN: Rink Resin = 0.308 gm

OPERATOR: A. Wong

RUN EDITOR:

Cy: 1 Rpt: 1 M: d

Cy: 2 Rpt: 14 M: BADEFG

Cy: 16 Rpt: 1 M: bdc

ADD TIME HOLD CYCLE: 0

RESIN SAMPLES: No

EVENTS LOG:		DATE	TIME	CYCLE	MOD	STEP	SEC REM
Synthesis begun		12/06/96	10:33	1	1	1	1
Barcode read	~~Asn~~ Tyr	12/06/96	10:45	2	2	1	9
Barcode read	Gly	12/06/96	11:38	3	2	1	9
Barcode read	Val	12/06/96	12:32	4	2	1	9
Barcode read	Asp	12/06/96	13:26	5	2	1	9
Barcode read	Glu	12/06/96	14:20	6	2	1	9
Barcode read	Ala	12/06/96	15:15	7	2	1	9
Barcode read	Phe	12/06/96	16:09	8	2	1	9
Barcode read	Phe	12/06/96	17:03	9	2	1	9
Barcode read	Val	12/06/96	17:57	10	2	1	9
Barcode read	Leu	12/06/96	18:52	11	2	1	9
Barcode read	Lys	12/06/96	19:46	12	2	1	9

Barcode read	Gln	12/06/96	20:40	13	2	1	9
Barcode read	His	12/06/96	21:35	14	2	1	9
Barcode read	Cys	12/06/96	22:29	15	2	1	9
Synthesis complete		12/06/96	23:53	16	3	49	0

SynthAssist™
Peptide Synthesis

Report type: Sequence
10/25/96 @ 8:01 AM
File name: Raso 5mer 10/25/96 Seq

Sequence editor:

Sequence	:	Val-Val-Ile-Ala-Thr-[NH2]
Comments	:	V. Raso 5mer 0.25 mmol scale Rink Resin=0.81mmol/gm subst=0.308gm N-Terminal Deprotect
Disulfide Bonds	:	0
Chemistry	:	Fmoc

Composition:

Sequence	:	[H]-Val-Val-Ile-Ala-Thr-[NH2]
Composition	:	C23 H44 N6 O6
Weight	:	500.6418
C-Terminal	:	NH2
N-Terminal	:	H
# of Residues	:	5
AA List	:	AA / Count

AA	Count
Ala	1
Ile	1
Thr	1
Val	2

Calculations:
(Calculation not updated)

Alzeimers peptide

Nacetyl

make Cys - met-val - gly - COOH

Boston Biomedical Research Institute
Peptide Synthesis Requisition Form
ABI 431A. Synthesizer

Alzheimer's pept.

Please complete the following form and submit it to Anna Wong for each peptide to be synthesized.

Date 10-24-96

Investigator Name: Vic Raso Ext# 316

Department:

Sequence (use 3 letter code):

H_2N- Val - Val - Ile - Ala - Thr - amide

-COOH

The Amino Acids in stock are:

Fmoc-L-Ala	Fmoc-Gln(Trt)	Fmoc-L-Leu	Fmoc-L-Ser(tBu)
Fmoc-L-Arg(Pmc)	Fmoc-L-Gln(OtBu)	Fmoc-L-Lys(Boc)	Fmoc-L-Thr(tBu)
Fmoc-Asn(Trt)	Fmoc-Gly	Fmoc-L-Met	Fmoc-L-Trp
Fmoc-L-Asp(OtBu)	Fmoc-L-His(Trt)	Fmoc-L-Phe	Fmoc-L-Tyr(tBu)
Fmoc-L-Cys(Trt)	Fmoc-L-Ile	Fmoc-L-Pro	Fmoc-L-Val)

Choose a Resin: Rink Resin (Amide) X HMP Resin (Acid) ____

Choose a Scale: 0.10mmole ____ (for 20 residue, assuming 80-90% cleavage recovery, yields about 150-175 mg crude peptide)

0.25mmole X (for 20 residue, assuming 80-90% cleavage recovery, yields about 390-440 mg crude peptide)

Do you want the N-Terminal Fmoc removed? Yes X No ____

Additional Instructions

Operators Comments Final test OK, started 10/25/96

Fees: Set-Up Charge $25.00
0.25mmole Scale $12.00/Amino Acid
0.10mmole Scale $10.00/Amino Acid

Cleavage of the peptide from the resin is not included in this service!!!

B001507

ABI MODEL 431A VERSION 2.00 Fmoc

SYNTHESIS REPORT

SEQUENCE NAME: V. Raso 5-mer 0.25 scale

RESIN: Rink Resin = 0.308 gm

OPERATOR: A. Wong

RUN EDITOR:

Cy: 1 Rpt: 1 M: d

Cy: 2 Rpt: 5 M: BADEFG

Cy: 7 Rpt: 1 M: bdc

ADD TIME HOLD CYCLE: 0

RESIN SAMPLES: No

EVENTS LOG:		DATE	TIME	CYCLE	MOD	STEP	SEC REM
Synthesis begun		10/25/96	09:17	1	1	1	1
Barcode read	Thr	10/25/96	09:29	2	2	1	9
Barcode read	Ala	10/25/96	10:22	3	2	1	7
Barcode read	Ile	10/25/96	11:16	4	2	1	9
Barcode read	Val	10/25/96	12:10	5	2	1	9
Barcode read	Val	10/25/96	13:04	6	2	1	9
Synthesis complete		10/25/96	14:27	7	3	49	0

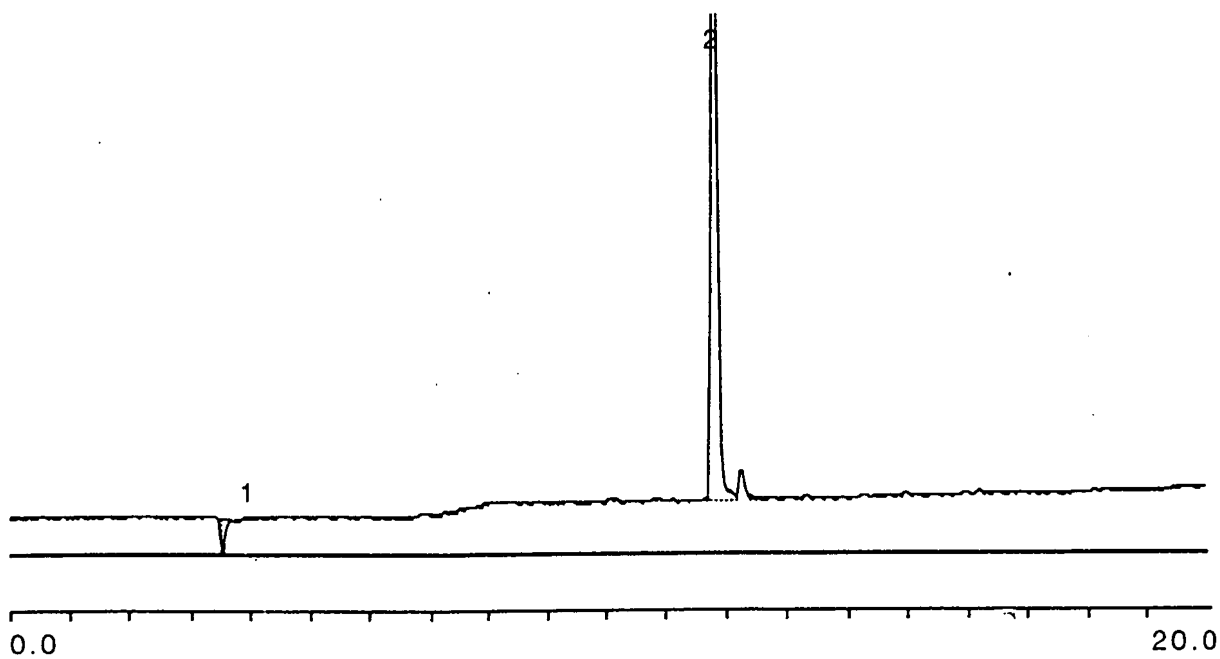
B001508

Date: Tue, Oct 29, 1996 3:00 PM
Data: pepanal 0-60-29OCT96-001

Sample: V. Raso 5mer 25µg injected
Column: C8 analytical 1ml/min
Buffer A: 0.1% TFA; B: 0.1%TFA in 90% CH3CN, 9.9% water
Gradient: 0-60%B, 20'
Monitor: 220nm, 2.0 AUFS; 280nm, 1.0 AUFS

Processing File: profile#1
Method: pepanal 0-60
Sampling Int: 0.1 Seconds

Data:



Analysis: Channel A

Peak No.	Time	Type	Height(µV)	Area(µV-sec)	Area%
1	3.938	N8	385	112003	5.720
2	11.800	N5	359251	1846084	94.279
Total Area				1958087	99.999

SynthAssist™ Peptide Synthesis

Report type: Sequence
7/8/96 @ 1:06 PM
File name: Raso 5merX3 7/8/96 Seq

√

Sequence editor:

Sequence : Leu-Met-Val-Gly-Gly-[NH2]

Comments : V. Raso 5mer X 3 0.25 scale
Rink resin=0.81mmol/gm subst=0.308gm
N-Terminal deprotect

Disulfide Bonds: 0
Chemistry : Fmoc

Composition:

Sequence : [H]-Leu-Met-Val-Gly-Gly-[NH2]

Composition : C20 H38 N6 O5 S1
Weight : 474.6239
C-Terminal : NH2
N-Terminal : H
# of Residues : 5
AA List :

AA	Count
Gly	2
Leu	1
Met	1
Val	1

Alzheimer peptide

Calculations:
(Calculation not updated)

B001510

Boston Biomedical Research Institute
Peptide Synthesis Requisition Form
ABI 431A Synthesizer

5 mg
x 3

Please complete the following form and submit it to Anna Wong for each peptide to be synthesized.

Date 7/3/96

Investigator Name: Vic Raso Ext# 326

Department:

Sequence (use 3 letter code):

H_2N- Leu - Met - Val - Gly - Gly - amide

-COOH

The Amino Acids in stock are:

Fmoc-L-Ala	Fmoc-Gln(Trt)	Fmoc-L-Leu	Fmoc-L-Ser(tBu)
Fmoc-L-Arg(Pmc)	Fmoc-L-Glu(OtBu)	Fmoc-L-Lys(Boc)	Fmoc-L-Thr(tBu)
Fmoc-Asn(Trt)	Fmoc-Gly	Fmoc-L-Met	Fmoc-L-Trp
Fmoc-L-Asp(OtBu)	Fmoc-L-His(Trt)	Fmoc-L-Phe	Fmoc-L-Tyr(tBu)
Fmoc-L-Cys(Trt)	Fmoc-L-Ile	Fmoc-L-Pro	Fmoc-L-Val)

Choose a Resin: Rink Resin (Amide) X HMP Resin (Acid) ____

Choose a Scale: 0.10mmole ____ (for 20 residue, assuming 80-90% cleavage recovery, yields about 150-175 mg crude peptide)

0.25mmole X (for 20 residue, assuming 80-90% cleavage recovery, yields about 390-440 mg crude peptide)

Do you want the N-Terminal Fmoc removed? Yes X No ____

Additional Instructions run 3-4 times

Operators Comments Flow Test OK, Started Batch I 7/16/96
Flow Test OK, Started Batch II 7/23/96 AM
Flow Test OK, Started Batch III 7/23/96 pm

Fees: Set-Up Charge	$25.00
0.25mmole Scale	$12.00/Amino Acid
0.10mmole Scale	$10.00/Amino Acid

Cleavage of the peptide from the resin is not included in this service!!!

B001511

ABI MODEL 431A VERSION 2.00 Fmoc

SYNTHESIS REPORT

SEQUENCE NAME: V Rnoo 5mer 0.25mmole

RESIN: Rink Resin = 0.36 gm

OPERATOR: A. Wong

RUN EDITOR:

Cy: 1 Rpt: 1 M: d

Cy: 2 Rpt: 5 M: BADEFG

Cy: 7 Rpt: 1 M: bdc

ADD TIME HOLD CYCLE: 0

RESIN SAMPLES: No

EVENTS LOG:	DATE	TIME	CYCLE	MOD	STEP	SEC REM
Synthesis begun	07/16/96	15:01	1	1	1	1
Barcode read Gly	07/16/96	15:13	2	2	1	9
Barcode read Gly	07/16/96	16:06	3	2	1	9
Barcode read Val	07/16/96	17:00	4	2	1	9
Barcode read Met	07/16/96	17:54	5	2	1	9
Barcode read Leu	07/16/96	18:48	6	2	1	9
Synthesis complete	07/16/96	20:11	7	3	49	0

ABI MODEL 431A VERSION 2.00 Fmoc

SYNTHESIS REPORT

SEQUENCE NAME: V Raso 5mer Batch II 0.25 mmole

RESIN: Rink Resin = 0.308gm

OPERATOR: A Wong

RUN EDITOR:

Cy: 1 Rpt: 1 M: d

Cy: 2 Rpt: 5 M: BADEFG

Cy: 7 Rpt: 1 M: bdc

ADD TIME HOLD CYCLE: 0

RESIN SAMPLES: No

EVENTS LOG:		DATE	TIME	CYCLE	MOD	STEP	SEC REM
Synthesis begun		07/23/96	09:35	1	1	1	1
Barcode read	Gly	07/23/96	09:47	2	2	1	9
Barcode read	Gly	07/23/96	10:41	3	2	1	9
Barcode read	Val	07/23/96	11:35	4	2	1	9
Barcode read	Met	07/23/96	12:29	5	2	1	9
Barcode read	Leu	07/23/96	13:23	6	2	1	9
Synthesis complete		07/23/96	14:46	7	3	49	0

ABI MODEL 431A VERSION 2.00 Fmoc

SYNTHESIS REPORT

SEQUENCE NAME: VPaso 5mer Batch III 0.25 mmole

RESIN: Rink Resin = 0.308 gm

OPERATOR: A. Wong

RUN EDITOR:

Cy: 1 Rpt: 1 M: d

Cy: 2 Rpt: 5 M: BADEFG

Cy: 7 Rpt: 1 M: bdc

ADD TIME HOLD CYCLE: 0

RESIN SAMPLES: No

EVENTS LOG:		DATE	TIME	CYCLE	MOD	STEP	SEC REM
Synthesis begun		07/23/96	16:13	1	1	1	1
Barcode read	Gly	07/23/96	16:24	2	2	1	9
Barcode read	Gly	07/23/96	17:18	3	2	1	9
Barcode read	Val	07/23/96	18:12	4	2	1	9
Barcode read	Met	07/23/96	19:06	5	2	1	9
Barcode read	Leu	07/23/96	20:00	6	2	1	9
Synthesis complete		07/23/96	21:23	7	3	49	0

SynthAssist™
Peptide Synthesis

Report type: Sequence
12/23/96 @ 9:43 AM
File name: Raso 4mer 12/23/96 Seq

√

Sequence editor:

Sequence : Val-Val-Ile-Ala-[NH2]

Comments : V. Raso 4mer 0.25scale
Rink Resin=0.78mmol/gm subst=0.320gm
N-Terminal Deprotect

Disulfide Bonds: 0
Chemistry : Fmoc

Composition:

Sequence : [H]-Val-Val-Ile-Ala-[NH2]

Composition : C19 H37 N5 O4
Weight : 399.5362
C-Terminal : NH2
N-Terminal : H
# of Residues : 4
AA List :

AA	Count
Ala	1
Ile	1
Val	2

3 batchs
A'2 peptide

Calculations:
(Calculation not updated)

Boston Biomedical Research Institute
Peptide Synthesis Requisition Form
ABI 431A Synthesizer

Please complete the following form and submit it to Anna Wong for each peptide to be synthesized.

Date 1-14-97

Investigator Name: Vic Raso Ext# 316

Department:

Sequence (use 3 letter code):

H2N- Val - Val - Ile - Ala

-COOH

The Amino Acids in stock are:

Fmoc-L-Ala	Fmoc-Gln(Trt)	Fmoc-L-Leu	Fmoc-L-Ser(tBu)
Fmoc-L-Arg(Pmc)	Fmoc-L-Glu(OtBu)	Fmoc-L-Lys(Boc)	Fmoc-L-Thr(tBu)
Fmoc-Asn(Trt)	Fmoc-Gly	Fmoc-L-Met	Fmoc-L-Trp
Fmoc-L-Asp(OtBu)	Fmoc-L-His(Trt)	Fmoc-L-Phe	Fmoc-L-Tyr(tBu)
Fmoc-L-Cys(Trt)	Fmoc-L-Ile	Fmoc-L-Pro	Fmoc-L-Val)

Choose a Resin: Rink Resin (Amide) X HMP Resin (Acid) ____

Choose a Scale: 0.10mmole ____ (for 20 residue, assuming 80-90% cleavage recovery, yields about 150-175 mg crude peptide)

0.25mmole X (for 20 residue, assuming 80-90% cleavage recovery, yields about 390-440 mg crude peptide)

Do you want the N-Terminal Fmoc removed? Yes X No ____

Additional Instructions run 3X

Operators Comments Flow test OK, Batch I started 1/16/97
Flow test OK, Batch II started 1/16/97
Flow test OK, Batch III started 1/17/97

Fees: Set-Up Charge	$25.00
0.25mmole Scale	$12.00/Amino Acid
0.10mmole Scale	$10.00/Amino Acid

Cleavage of the peptide from the resin is not included in this service!!!

ABI MODEL 431A VERSION 2.00 Fmoc

SYNTHESIS REPORT

SEQUENCE NAME: V Raso 4 mer 0.25 scale

RESIN: Rink Resin - 0.32 gm

OPERATOR: A. Wong

RUN EDITOR:

Cy: 1 Rpt: 1 M: d

Cy: 2 Rpt: 4 M: BADEFG

Cy: 6 Rpt: 1 M: bdc

ADD TIME HOLD CYCLE: 0

RESIN SAMPLES: No

EVENTS LOG:		DATE	TIME	CYCLE	MOD	STEP	SEC REM
Synthesis begun		01/16/97	10:32	1	1	1	1
Barcode read	Ala	01/16/97	10:44	2	2	1	9
Barcode read	Ile	01/16/97	11:38	3	2	1	9
Barcode read	Val	01/16/97	12:32	4	2	1	9
Barcode read	Val	01/16/97	13:26	5	2	1	9
Synthesis complete		01/16/97	14:49	6	3	49	0

ABI MODEL 431A VERSION 2.00 Fmoc

SYNTHESIS REPORT

SEQUENCE NAME: V. Raso 4 mer 0.25 mmole

RESIN: Rink Resin = 0.320 gm

OPERATOR: A. Wong

RUN EDITOR:

Cy:	1	Rpt:	1	M:	d
Cy:	2	Rpt:	4	M:	BADEFG
Cy:	6	Rpt:	1	M:	bdc

ADD TIME HOLD CYCLE: 0

RESIN SAMPLES: No

EVENTS LOG:		DATE	TIME	CYCLE	MOD	STEP	SEC REM
Synthesis begun		01/16/97	15:00	1	1	1	1
Barcode read	Ala	01/16/97	15:11	2	2	1	9
Barcode read	Ile	01/16/97	16:05	3	2	1	9
Barcode read	Val	01/16/97	16:59	4	2	1	9
Barcode read	Val	01/16/97	17:53	5	2	1	9
Synthesis complete		01/16/97	19:16	6	3	49	0

ABI MODEL 431A VERSION 2.00 Fmoc

SYNTHESIS REPORT

SEQUENCE NAME: V. Raso 4 mer 0.25 scale

RESIN: Rink Resin = 0.320 gm

OPERATOR: A. Wong

RUN EDITOR:

Cy: 1 Rpt: 1 M: d

Cy: 2 Rpt: 4 M: BADEFG

Cy: 6 Rpt: 1 M: bdc

ADD TIME HOLD CYCLE: 0

RESIN SAMPLES: No

EVENTS LOG:		DATE	TIME	CYCLE	MOD	STEP	SEC REM
Synthesis begun		01/17/97	10:17	1	1	1	1
Barcode read	Ala	01/17/97	10:29	2	2	1	9
Barcode read	Ile	01/17/97	11:23	3	2	1	9
Barcode read	Val	01/17/97	12:17	4	2	1	9
Barcode read	Val	01/17/97	13:11	5	2	1	9
Synthesis complete		01/17/97	14:34	6	3	49	0

11-5-96

Coupling the Alz-statene peptide MW ~1,000 to KLA and OVA

MW = 1X106

KLH 1mg = 12 mg solid $\frac{1\,mg}{1\times10^6} = 1\times10^{-9}$ moles × 172 mol groups
MW = 1X106
$= 1.7\times10^{-7}$ moles

use 0.172 mg peptide = equimolar

use 2 mg = 10X ~~excess~~ 1.7×10^{-6} moles

assume 50% pure use 3 mg = 10X ~~excess~~

OVA 1 mg = 6.5 mg $\frac{1\,mg}{4.5\times10^4} = 2.2\times10^{8}$ × 10 mol groups
MW = 45,000
$= 2\times10^{7}$ moles groups

use 4 mg peptide = ~10X excess

K

SH
|
C9O [illegible]

4,545
5,455 [illegible]

B001520

Immunizing Mice

11/8/96

- used Alz Sta KLH for antigen (100 uL)
- used 600 uL of PBS

***Draw these contents up first into the syringe (usually a 23 gauge needle)

- Then draw up 700 uL of complete Freund's Adjuvent separately, but into the same syringe.
- Put on syringe the double ended needle and attach another syringe. Be sure to wear goggles, lab coat and gloves. Push syringes back and forth to create emulsion. The consistency should get thick. When the consistency is right it is ready for injection.
- Inject each mouse IP

**Make sure the double ended needle is washed with ethanol first and then rinsed thoroughly with dH2O.

Mice Records

Date	Number of mice	Contents injected	Other Information
12/19/96	5	.5 mL protective antigen, .7 mL IFA. .2 mL PBS	delivered @ 200 uL into each mouse
	5 Ab Sta KLH	200 uL in CFA 11/8/96	200 uL in IFA 12/19/96
1/2/97	5	pristane	
3/11/97	5	pristane 3/20 Ab Sta 11E9, 5C7, Ab PS5A11; 3/6 fusion 5F6	
pooled mice 3/20/97	5	2 rt ear w/ KLH Mopc 167 2 lft ear w/ Mopc 167	1 mouse w/ .5mL KLH AZO phosphocholine
4/15/97	1 KLH AZO phos.	20λ KLH AZO + 250λ PBS + 250λ IFA	
"	2 KLH Mopc167 Fab	50λ KLH Mopc 167 Fab + 200λ PBS + 250λ IFA	
"	2 Mopc 167	100λ Mopc 167 + 200λ PBS + 300λ IFA	
5/20/97	1	pristane 5/23/97 HGH (Mopc 167)	for ascites production
5/21/97	2 (rt ear)	pristane	

Pristane Prime Mice

Use ⓐ .5 mL of Pristane (clear)
Draw up w/ 18 g.
& inject with another smaller syringe

B001522

Immunizing Mice

11/8/96

* used eppendorf tube & then cut in half to take up contents!

Used A12 Sta KLH for antigen (100 µL)
Used 600 µL PBS
} draw into syringe 1st

Use a 25g needle *

Then draw up 700 µL complete freund's adjuvant separately but into same syringe

put on syringe the double ended needle & attach another syringe
(wear gloves goggles & lab coat)
push back & forth to create emulsion
when consistency is thick it is ready for injection

Inject each mouse IP

* make sure double ended needle is washed w/ ethanol 1st then rinsed COMPLETELY w/ Distilled water.

1996

Elisa Results from A1z Clones

11/11/96

A12

	1	2	3	4	5	6	7	8	9	10	11	12	
A	Hy	1A1	1A2	1A4	1A5	1A6	1A7	1A8	+- 1A9	1A10	1A11	1A12	10 uL
B	1B1	1B2	1B3	1B4	1B5	+ 1B6	1B7	1B8	1B9	1B10	1B11	+ 1B12	90 uL H
C	+ 1C1	+ 1C2	1C3	1C4	1C5	1C6	1C7	1C8	1C9	1C10	1C11	1C12	50 uL
D	1D1	1D2	1D3	1D4	1D5	1D6	1D7	1D8	+ 1D9	1D10	1D11	1D12	
E	Hy	+ 1B6	+ 1C1	+ 1C2	+ 1D9	1E6	1E7	1E8	1E9	1E10	1E11	1E12	
F	1F1	1F2	1F3	+ 1F4	+ 1F5	1F6	+ 1F7	1F8	1F9	1F10	1F11	1F12	
G	1G1	1G2	1G3	1G4	1G5	1G6	1G7	1G8	+ 1G9	1G10	1G11	1G12	
H	+ 1H1	+ 1H2	1H3	1H4	1H5	1H6	+ 1H7	1H8	1H9	+ 1H10	1H11	1H12	

Rows C+D

All clones showed up +, but 3 wells were darker than the rest

~~Multiclones to Be Separated:~~

1B6, 1C1, 1C2, 1D9, 1F4, 1F5, 1F7, 1G9, 1H1, 1H2, 1H7, 1H10

11/20/96

β amyloid 1-43

	1	2	3	4	5	6	7	8	9	10	11	12
A	H4	+cont 2D8 +	-cont 2E3 +	1H2	1H7							
A12 fusion B												
C												
D												
E												
F												
G												
H												

	1	2	3	4	5	6	7	8	9	10	11	12
3A	0.027	0.070	0.024	0.024	0.028	-0.000	-0.001	-0.001	-0.001	-0.001	-0.000	-0.001
3B	-0.000	-0.000	-0.001	0.000	0.000	-0.001	-0.000	0.001	-0.001	-0.001	-0.001	-0.000

END OF RUN

	1	2	3	4	5	6	7	8	9	10	11	12
4A	0.026	0.069	0.026	0.023	0.027	-0.002	-0.001	-0.002	-0.001	-0.001	-0.003	-0.002

END OF RUN
TEST ENDED

ABSORBANCE MODE 12 PAGE 1 11/20/96 13:46:46
LOT NUMBER:________ EXP. DATE:________ USER:________
WAVELENGTHS=450NM 630NM

	1	2	3	4	5	6	7	8	9	10	11	12
1A	0.151	3.369	1.623	0.105	0.119	-0.000	-0.000	-0.001	0.000	-0.000	0.000	0.000

END OF RUN
TEST ENDED

ABSORBANCE MODE 12 PAGE 1 11/20/96 13:48:18
LOT NUMBER:________ EXP. DATE:________ USER:________
WAVELENGTHS=405NM 630NM

	1	2	3	4	5	6	7	8	9	10	11	12
1A	0.087	1.606	0.740	0.069	0.076	-0.003	-0.003	-0.002	-0.002	-0.003	-0.003	-0.002
A12 fusion	1H4	2D8	2E3	1H12	1H7							

12/30/96

↙ A12 ps

Comparing A12 Clones on A12-Sta plate + β amyloid 1-43

	1	2	3	4	5	6	7	8	9	10	11	12	
A	HY	5G4	6E2	8E6	2F1	1H2	3B1	4B11	8D12	4G7	8E3	8E3	A12
B	gr 2E3	red 3C1(1)	red 4H9	red 5A11	vio 6F11	vio 2C11	vio 4C7	gr 5A3	gr 2C12	gr 5C7	gr 2H11	gr 7E4	
C	HY	5G4	6E2	8E6	2F1	1H2	3B1	4B11	8D12	4G7	8E3	8E3	β amy 1-43
D	2E3	3C1(1)	4H9	5A11	6F11	2C11	4C7	5A3	2C12	5C7	2H11	7E4	
E													
F													
G													
H													

A12 ⊕

5G4	2E3
6E2	3C1(1)
8E6	4H9
1H2	5A11
3B1	6F11
8D12	2C11
4G7	4C7
8E3	2C12
8E3	5C7
	2H11
	7E4

β amyloid 1-43 ⊕

6E2 *
5A11 ?
6F11 ?
2E3 ?

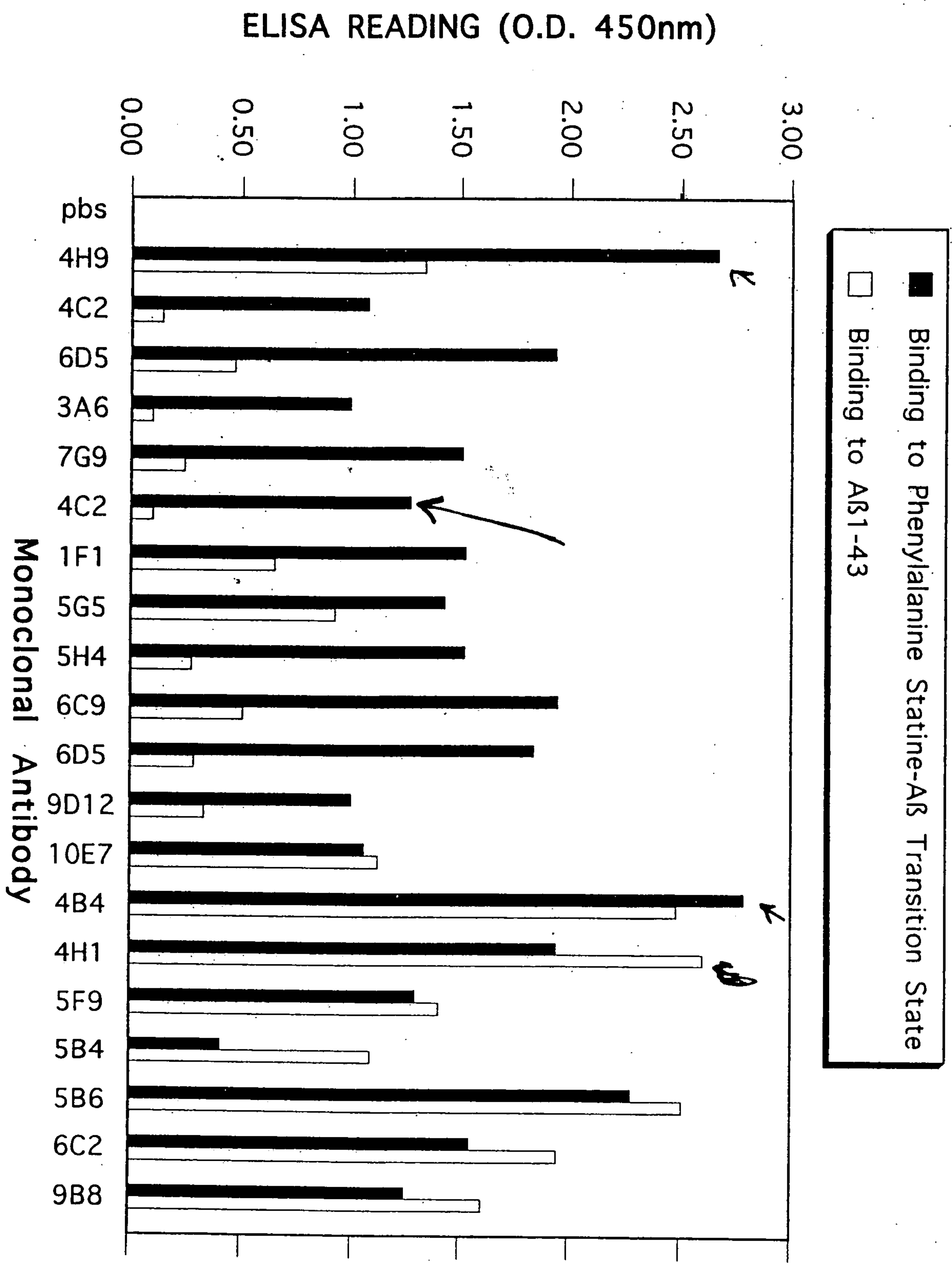
1LZ
P5

STAT FAX 2100 :S SN 1112 12/30/96 16:28:04
ABSORBANCE MODE 12 PAGE 1 12/30/96 16:29:19
LOT NUMBER:______ EXP. DATE:______ USER:______
WAVELENGTHS=450NM 630NM

	1	2	3	4	5	6	7	8	9	10	11	12
1A	0.045	1.633	2.866	1.695	0.058	1.913	1.580	1.637	0.058	1.935	2.229	•2.366
	Hy	5G4	·6E2	8E6	2F1	1H2	3B1	8D12	4B11	4G7	8E3	8E3
1B	•2.383	1.098	2.079	•2.391	•1.661	2.180	1.678	0.116	2.722	2.101	1.598	•2.066
	2E3	3C1(1)	4H9	5A11	~~6F11~~	~~2C11~~	~~4C7~~	~~5A3~~	~~2G12~~	~~5C7~~	~~2H11~~	~~7E4~~
1C	0.066	0.069	2.400	0.072	0.074	0.065	0.050	0.073	0.030	0.047	0.091	•0.106
B1-43	Hy	5G4	·6E2	8E6	2F1	1H2	3B1	4B11	8D12	4G7	8E3	8E3
1D	0.409	0.047	0.066	•1.568	•0.950	0.097	0.035	0.087	0.056	0.067	0.038	•0.111
	2E3	3C1(1)	4H9	5A11	6F11	2C11	4C7	5A3	2G12	5C7	2H11	7E4
1E	-0.000	-0.001	-0.000	-0.000	-0.000	0.000	-0.000	-0.000	0.000	-0.000	-0.000	0.000

END OF RUN

X Rx 6E2
5A11
6F11
2E3

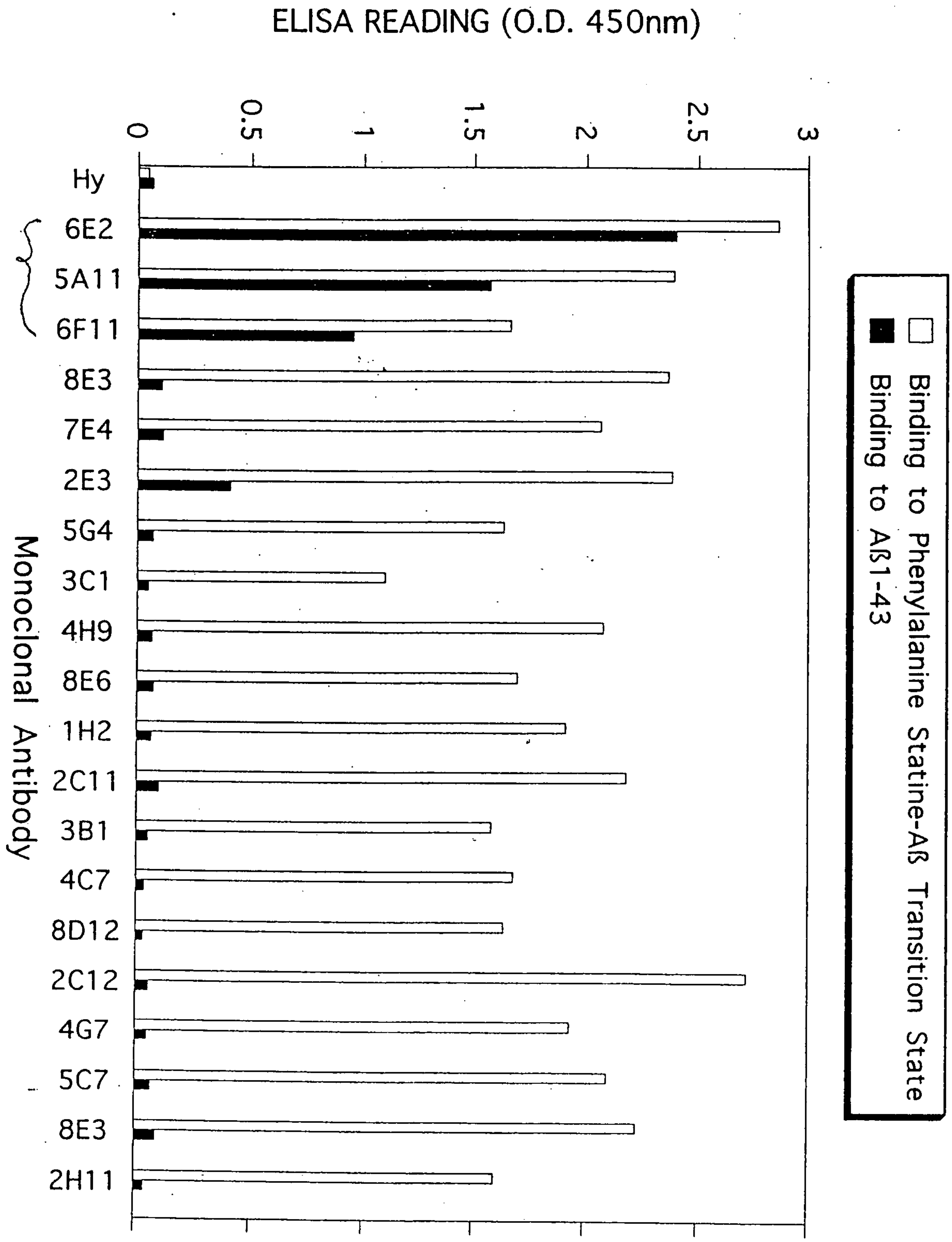


1 609-290-2088

12/31/96

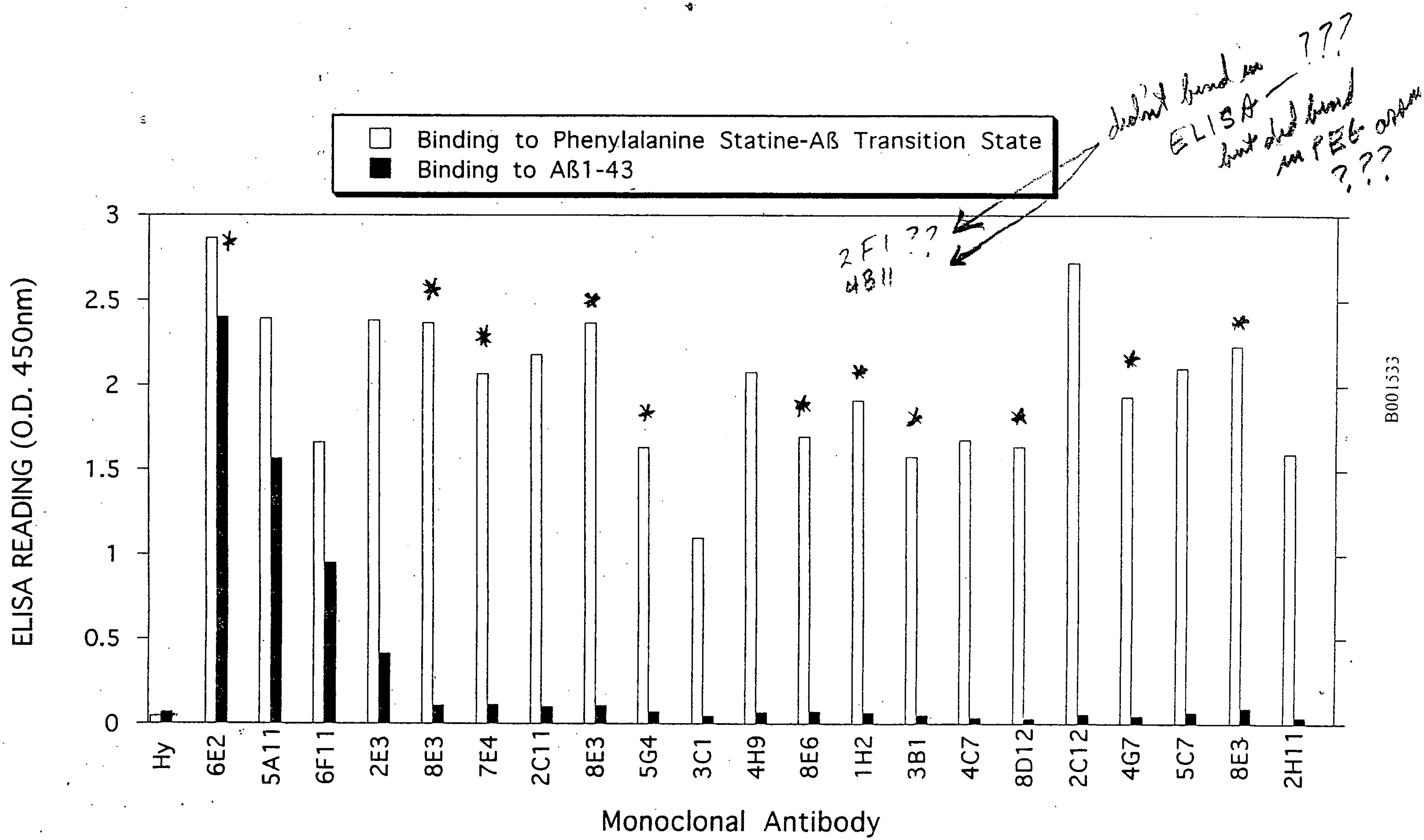
	1	2	3	4	5	6	7	8	9	10	11	12	
A	Hy	2F1	4B11	5A3	3B1	3H10	6D12	2C11	8F1	1H7	3G1	8E4	β amyl 1-43
B	Hy	2F1	4B11	5A3	+ 3B1	+ 3H10	6D12	+ 2C11	8F1	1H7	+ 3G1	8E4	A12 Sta
C													
D													
E													
F													
G													
H													

* testing A12 clones on β-amyloid 1-43 & A12 Sta plates



ELISA READING (O.D. 450nm)
0
0.5
1
1.5
2
2.5
3
Monoclonal Antibody
Hy
6E2
5A11
6F11
8E3
7E4
2E3
5G4
3C1
4H9
8E6
1H2
2C11
3B1
4C7
8D12
2C12
4G7
5C7
8E3
2H11
Binding to Phenylalanine Statine-Aß Transition State
Binding to Aß1-43

B001532


Binding to Phenylalanine Statine-Aß Transition State
Binding to Aß1-43
ELISA READING (O.D. 450nm)
0
0.5
1
1.5
2
2.5
3
Hy
6E2
5A11
6F11
2E3
8E3
7E4
2C11
8E3
5G4
3C1
4H9
8E6
1H2
3B1
4C7
8D12
2C12
4G7
5C7
8E3
2H11
Monoclonal Antibody
2F1 ??
4B11
didn't bind in ELISA — ???
but did bind in PEG assay
???

B001533

11/21/96

Alz

	1	2	3	4	5	6	7	8	9	10	11	12
A	Hy	+/- 1H1	+ 1H2	+/- 1H7	+ 2C11	+ 2C2	+ 2D8	+ 2E3	2F1	+ 2F2	+ 2H6	+ 2H11
B	+ 3B11	+ 3B1	+ 3D5	+ 3C1	+ 3G7							
C												
D												
E												
F												
G												
H												

Testing the clones that were tested on β-amyloid 1-43 to see if still reactive

TEST ENDED

ABSORBANCE MODE 12 PAGE 1 11/21/96 14:26:43

LOT NUMBER:__________EXP. DATE:__________USER:__________

WAVELENGTHS=450NM 630NM

	1	2	3	4	5	6	7	8	9	10	11	12
1A	0.102	0.251	1.489	0.444	1.708	1.862	1.508	1.491	0.199	0.996	1.516	1.069
		1H1	1H2	1H7	2C11	2C12	2D8	2E3	2F1	2F2	2H6	2H11
1B	1.467	1.217	1.725	1.119	1.584	0.000	0.000	-0.000	-0.000	-0.001	0.000	0.000
	3A11	3B1	3B5	3C1	3G1							
1C	-0.000	0.000	-0.000	-0.000	0.000	-0.000	0.000	0.000	0.000	0.000	0.000	0.000

END OF RUN

TEST ENDED

ABSORBANCE MODE 12 PAGE 1 11/21/96 14:28:44

LOT NUMBER:__________EXP. DATE:__________USER:__________

WAVELENGTHS=492NM 630NM

	1	2	3	4	5	6	7	8	9	10	11	12
1A	0.045	0.118	0.667	0.201	0.769	0.834	0.686	0.672	0.089	0.453	0.696	0.481
1B	0.656	0.547	0.782	0.506	0.718	-0.000	0.000	0.000	0.000	0.000	0.000	0.000
1C	-0.001	-0.001	-0.000	-0.001	-0.001	-0.000	-0.001	0.000	-0.001	0.000	-0.000	-0.000

END OF RUN

11/10/96

Bamyloid 1-43

R12 fusion

	1	2	3	4	5	6	7	8	9	10	11	12
A	1H1	2C11	2C12	+ 2E3	2F2	2H6	+/- 3B1	3C1	3G1			
B												
C												
D (11/9/96)	+ 3D8	2F1	2H11	3A11	+ 3B1	3B5						
E												
F												
G												
H												

B001536

11/22/14

	1	2	3	4	5	6	7	8	9	10	11	12	
A	4B4	4H1	4H9	5F9	4C2	5B4	5B6	6C2	6D5	9B8	9D12	3A6	V_3 loop
B	3C11	3E5	3G7	2H4	1C4	5E1	5G10	6F6	6F7	7G9	10E6	10E7	
C	1C4	3C11	4C2	4H1	1D1	1F1	5G5	5H4	6C9	6D5	9D12	10E7	
D													
E		SAME AS ABOVE											PSII
F													
G													
H													

* testing PSII clones on both plates.

A/2
11/22/96

	1	2	3	4	5	6	7	8	9	10	11	12
A						8B8	8D8	+ 8D8	+ 8C11	8C11	8C11	8C11
B	8C11	+ 8D8	8D8	8D12	+ 8D12	8E3	+ 8E3	+ 8E4	8E4	8E4	+ 8E6	8E6
C	+ 8F1	8F1	8F6	+ 8F6	8F6	8F6	+ 8G12	8G12	+ 8H6	8H6	9B10	+ 9B10
D												
E												
F												
G												
H												

* testing the separated clones

A12[S] clones

	1	2	3	4	5	6	7	8	9	10	11	12 flasks
A (microtiter)	Rewc 2E3	2E3 +	2H11 +/-	2H11	4H9	4H9 ±	5A3	5A3 ±	3B1 +/-	3B1	4H9	4H9 w/CS
B (microtiter)	Rew D 2E3 +	2E3+	2H11 +/	2H11	4H9	4H9	5A3	5A3 ±	3B1	3B1	5A3	3B1
C flasks	2H11	w/CS 2E3+	w/CS 5A3	2E3 +	w/CS 2H11	Hy						
D												
E												
F												
G												
H												

Tested microtiter wells + flasks for rescued clones

Results

2E3 + more positive in the microtiter plate than in the flasks

2H11 +/-

Neg 4H9

ELISA 11/24/97

A/z PS plate

1 Hy
2 Hy
3 Hybrid 10λ
4 1λ
5 BAll 65mg 1λ
6 .1λ
7 BAll 40mg 1λ
8 .1λ

BT rxn
Hy 225λ
100λ/tube

B001540a

STAT FAX 2100 :S SN 1112 11/25/97 08:27:51
ABSORBANCE MODE 12 PAGE 1 11/25/97 08:27:58
LOT NUMBER:______ EXP. DATE:______ USER:______

WAVELENGTHS=450NM 630NM

	1	2	3	4	5	6	7	8	9	10	11	12
1A	0.391	0.676	1.743	1.551	1.033	0.823	0.849	0.863	0.001	0.002	0.001	0.002
1B	0.001	0.001	0.002	0.002	0.000	0.001	0.002	0.002	0.001	0.001	0.001	0.001

ELISA 11/24/97

A/z PS plate

1 Hy
2 Hy
3 Hybrid 10λ
4 1λ
5 BA11 65mg 1λ
6 .1λ
7 BA11 40mg 1λ
8 .1λ

Ab 100
Hy 2.5λ
100λ 1/100λ

B001540a

STAT FAX 2100 :S SN 1112 11/25/97 08:27:51
ABSORBANCE MODE 12 PAGE 1 11/25/97 08:27:58
LOT NUMBER:_______________EXP. DATE:_______________USER:_______________

WAVELENGTHS=450NM 630NM

	1	2	3	4	5	6	7	8	9	10	11	12
1A	0.391	0.676	1.743	1.551	1.033	0.823	0.849	0.863	0.001	0.002	0.001	0.002
1B	0.001	0.001	0.002	0.002	0.000	0.001	0.002	0.002	0.001	0.001	0.001	0.001

11/20/96

A12

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F							6H1 1	+ 6H1 2	+ 7C11 1	7C11 2	7D10 1	+ 7D10 2
G	+ 7E4 1	7E4 2	7E12 1	+ 7E12 2	+ 7F2 1	7F2 2	7F2 3	7F9 1	+ 7F9 2	+ 7F10 1	7F10 2	7F10 3
H												

* testing separated clones

"1'"1'4

A12

	1	2	3	4	5	6	7	8	9	10	11	12
A	Ny	1D9 1	1D9 2	1D9 3	2B4 1	2B4 2	2B4 3	2E11 1	2E11 2	4B11 1	4B11 2	⊕ 4B11 3
B	4C7 1	⊕ 4C7 2	4C7 3	4D5 1	⊕ 4D5 2	⊕ 4G7 1	4G7 2	4G7 3	4G7 4	4G7 5	4H1 1	4H1 2
C	4H1 3	4H1 4	4H1 5	4H9 1	⊕ 4H9 2	4H9 3	4H9 4	5A3 1	5A3 2	⊕ 5A3 3	5A3 4	5A11 1
D	5A11 2	⊕ 5A11 3	5C7 1	⊕ 5C7 2	5C7 3	⊕ 5D2 1	5D2 2	5D2 3	5D2 4	5F3 1	⊕ 5F3 2	5G4 1
E	⊕ 5G4 2	⊕ 5G11 1	5G11 2	⊕ 6A1 1	6A1 2	⊕ 6D12 1	6D12 2	6E2 1	6E2 2	⊕ 6E2 3	⊕ 6E6 1	6E6 2
F	6E6 3	⊕ 6F11 1	6F11 2	6G5 1	6G5 2	⊕ 6G5 3						
G												
H												

* testing separated clones
retesting 1D9, 2B4, + 2E11

No reaction:

1D9
2B4
2E11
4H1

A12
11/15/96
plate#9

	1	2	3	4	5	6	7	8	9	10	11	12
A	9A1	9A2	9A3	9A4	9A5	9A6	9A7	9A8	9A9	9A10	9A11	9A12
B	9B1	9B2	9B3	9B4	9B5	9B6	9B7	9B8	9B9	+ 9B10	9B11	9B12
C	9C1	9C2	9C3	9C4	9C5	9C6	9C7	9C8	9C9	9C10	9C11	9C12
D												
E												
F												
G												
H												

R12
11/15/96
plate #8

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B								+ 8B8				
C											+ 8C11	
D								+ 8D8				+ 8D12
E			+ 8E3	+ 8E4		+ 8E6						
F	+ 8F1					+ 8F6						
G												+ 8G12
H						+ 8H6						

11/15/94

ALZ

plate # 7

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C											⊕ 7C11	
D										⊕ 7D10		
E				⊕ 7E4								⊕ 7E12
F		⊕ 7F2							⊕ 7F9	⊕ 7F10		
G												
H												

A12
11/15/96

	1	2	3	4	5	6	7	8	9	10	11	12
A	+ 1B6 1	1B6 2	1C1 1	+ 1C1 2	1C1 3	+ 1C2	1C2 2	1D9 1	1D9 2	1D9 3	+ 1F4 1	1F4 2
B	1F4 3	1F5 1	+ 1F5 2	1F5 3	1F5 4	1F5 5	1F7 1	1F7 2	+ 1F7 3	1F7 4	1F7 5	+ 1G9 1
C	1G9 2	1G9 3	1G9 4	1H1 1	1H1 2	+ 1H1 3	1H2 1	+ 1H2 2	+ 1H7 1	1H7 2	1H7 3	1H7 4
D	1H10 1	1H10 2	1H10 3	+ 1H10 4	2B4 1	2B4 2	2B4 3	2B5 1	2B5 2	2B5 3	+ 2B5 4	2C11 1
E	[illegible] 2C11 2	2C11 3	+ 2C11 4	+ 2C12 1	2C12 2	2C12 3	2D8 1	2D8 2	+ 2D8 3	2E3 1	2E3 2	2E3 3
F	2E3 4	+ 2E3 5	2E11 1	2E11 2	2F1 1	2F1 2	2F1 3	+ 2F1 4	2F2 1	+ 2F2 2	2H6 1	2H6 2
G	+ 2H6 3	2H6 4	2H11 1	2H11 2	2H11 3	+ 2H11 4	3A11 1	+ 3A11 2	3B1 1	3B1 2	+ 3B1 3	3B1 4
H	+ 3B5 1	3B5 2	3B5 3	3B5 4	+ 3C1 1	3C1 2	3G1 1	+ 3G1 2	3H10 1	3H10 2	+ 3H10 3	3H10 4

Testing the separated clones

Retest:
1D9
2B4
2E11

plate #6

	1	2	3	4	5	6	7	8	9	10	11	12
A	+ 6A1											
B												
C												
D												+ 6D12
E		+ 6E2				+ 6E6						
F											+ 6F11	
G					+ 6G5							
H	+ 6H1											

plate #5

	1	2	3	4	5	6	7	8	9	10	11	12
A			+ 5A3								+ 5A11	
B												
C							+ 5C7					
D		+ 5D2										
E												
F			+ 5F3									
G				+ 5G4							+ 5G11	
H												

B001548

ALZ
plate #4

	1	2	3	4	5	6	7	8	9	10	11	12
A	4A1											
B	4B1										+ 4B11	
C	4C1						+ 4C7					
D	4D1				+ 4D5							
E	4E1											
F	4F1											
G	4G1						+ 4G7					
H	+ 4H1								+ 4H9			

A12
11/12/96

	1	2	3	4	5	6	7	8	9	10	11	12
A	3A1										+ 3A11	3A12
B	+ 3B1				+ 3B5							
C	+ 3C1											
D	3D1											
E	3E1											
F	3F1											
G	+ 3G1											
H	3H1									+ 3H10		

Multiclones were separated on 11/12/96

#12
11/12/96

	1	2	3	4	5	6	7	8	9	10	11	12
A	2A1											2A12
B	2B1			+ 2B4	+ 2B5							
C	2C1										+ 2C11	+ 2C12
D	2D1							+ 2D8				
E	2E1		+ 2E3								+ 2E11	
F	+ 2F1	+ 2F2										
G	2G1											
H	2H1					+ 2H6					+ 2H11	

Multiclones were separated on 11/12/96

STAT FAX 2100 :S SN 1112 01/23/97 16:09:16

ABSORBANCE MODE 12 PAGE 1 01/23/97 16:09:24

LOT NUMBER:________ EXP. DATE:________ USER:________

WAVELENGTHS=450NM 630NM

	1	2	3	4	5	6	7	8	9	10	11	12
1A	-0.000	-0.000	0.235	0.273	0.252	0.669	0.109	0.434	1.983	0.895	1.694	1.394
			1Hy	3F2	2H03	5C7	6D9	6M6	6E2(1:2)	(mut1:2)	6E2(un)	(mut)(un)
1B	-0.000	0.000	0.000	0.001	0.000	-0.000	-0.000	0.000	-0.000	-0.000	-0.000	-0.000

END OF RUN

B001552

1/13/97

	1	2	3	4	5	6	7	8	9	10	11	12	
A			4Y	3G4 + cont	6D12	3B1	8E6	8E4 (2)	8E4 (1)	6F11 (2)	4G7	5H11 (2)	Alz (PS)
B	3B5	8E3	6E6	6F11 (1)									
C													
D	4Y	6D12	3B1	8E6	8E4 (2)	8E4 (1)	+ 6F11 (2)	4G7	3B5	8E3	+ 6E6	+ 6F11 (1)	B amyloid 1-43
E	5H11 + (2)	6E2 + cont											
F													
G													
H													

testing Alz flasks for antibody production

Results:

Alz

++ 3B5
++ 8E3
++ 6E6
++ 6F11(1)
+ 8E6
+ 6F11(2)
+/- 3B1
+/- 4G7
+/- 5H11(2)

B amyloid 1-43

+ 6F11(2)
+ 6E6
+ 6F11(1)
+/- 5H11(2) → already injected into mice

B001553

H12 PS

	1	2	3	4	5	6	7	8	9	10	11	12
A	Hy	8E4 (1) 19.f1.	8E4 (2) 19.f1.	+/- 8E6 19.f1.	+/- 6F11 19f1.	+/- 3B1	+ 6E6	+ 6E2	+ 4H9	+ 3B7	+/- 5H11	+/- 8E3
B	+ 6F11	6D12	+ 5H11	+/- 6F11	+ or +/- 6E2	+/- or - 5G4						
C												
D												
E												
F												
G												
H												

infected 6E2
4H9 look bad
5A11 look bad

5G4
8E6
8D12
8E3
4G7

1/14/97
#12

	1	2	3	4	5	6	7	8	9	10	11	12
A	H4	4F11 2D++	4F11 [illegible]+	9E6 D1++	9E2 [illegible]++	7G7 6C++	[illegible] [illegible]					
B												
C												
D												
E												
F												
G												
H												

testing single clones from plates
(Trying to reclone wells from flasks)
Very few single clones on plates

Results:

all were (+)

1/10/97:
plate clones in limiting dilution

1/7/97

		1	2	3	4	5	6	7	8	9	10	11	12
Alz	A	Black	Blue	Yellow	Red +	Green	cont.						
	B												
Alz Sta	C	Black +	Blue +	Yellow +	Red +	Green +	cont.						
	D												
	E												
	F												
	G												
	H												

* Tested serum from Alz Sta mice

~ 5 hours after i.v. injection 11/29/96 Friday

	wt	cpm - Bkg	cpm/gm	B/B - Bkg
		~ 60		
blood	23.8 mg	1,976	83.0 × 10^3	1.0
brain	387.5 mg	1,094	2.8 × 10^3	0.034
spleen	109.6 mg	8,840	80.7 × 10^3	0.97
kidney	171.2 mg	14,890	87.0 × 10^3	1.05
heart	113.3 mg	3,150	27.8 × 10^3	0.33
liver	376.4 mg	71,872	190.9 × 10^3	2.3
muscle	101.7 mg	1,544	15.2 × 10^3	0.18

37361
36228
133

37854
36142
.1712

3.7311
36315
.1096

40079
36315
.3764

37177
36160
1017

3.6592
3.6334
238

4.0180
3.6305
.3875

brain/blood

$2.8 \times 10^3 / 83 \times 10^3 =$

3.4×10^{-2}

37 × ~~1000~~

B001557

I.V. injection of immunized ... (green) AL2 ... statine   bkg (60)   cpm/gm   (- Bkg)

		cpm		B/B
blood	0.1236 gm	2,203	17.8×10^3	1.0
brain	.4385	330 - 57	0.75×10^3	.042 (0.62×)
spleen	.1660	2,402	14.4×10^3	0.81
kidney	.2550	5,442	21.3×10^3	1.2
heart	.1685	1,398	8.3×10^3	0.47
liver	.3757	10,012	26.6×10^3	1.49
muscle	.1623	456	2.8×10^3	0.16

blood
3.7764
3.6528
.1236

brain
39906
35521
4385

spleen 38053
36393
1660

liver 4.0204
36447
.3757

$$B/B \quad \frac{0.75 \times 10^3}{17.8 \times 10^3} = 4.2 \times 10^{-2}$$
42 × 1000

kidney 38905
36355
.2550

$$\frac{0.62}{17.3} = 35 \times 1000$$

heart 37417
35737

muscle
38018
36395
.1623

B001559

43) Alz 1-40 pg 89

44) 5B11 A-Id pg 91

for Alzheimer's

Human

45) HUV-EC-C umbilical cord endothelial cells p 93

46) 293 Human embryonic kidney Adenovirus transform p 95

not umbilical cord → 47) ECV 304 pg 97 ← this ~~was~~ is a T-24 - human bladder cancer

48) SY5Y pg 99

49) COKE p 101 plus 2 KW clones

* 50) HTB-20 p 103 = BT-474 overexpresses c-er

51) KW p 105

52) SP2/0 p 107

53) P3 p 109 (NS-1)

54) Alz FF p 111

55) Alz Sta II p 113

56) Alz FX p 115

57) Alz KL p 117

58) Alz XF p 119

59) Alz KL super p 121

60) EOC-2 microglial p 123

61) TMB1 p 125

62) B3X63 p 127

B001560

65) Alz II p. 137

66) KL3 clones p. 141

67) KLH-Hep4 clones p. 139.

68) NS-1 clones p. 135.

69) XH-II clones p. 147.

70) MN p. 143.

71.

73. p. 149.

74. p. ~~[illegible]~~ 131

COKE	Alz FF
NS-1	Alz XH-II
SP2	Alz PS
TMB-1	Alz Q Sta.
EOC2	Alz Sta. II
P3x63	Alz KL Super
CEM	Alz KL
COS-7	KL3
MN	KLH Hep 4
HN	P3C Hep
KW	Alz FX
HTB-20	Alz XF
cerb	Alz II
DT	SP02-CRL

* * now in cell freezing IV book *

3J - 1 vial

3G 1 vial

9/8/99 COKE 10G5
3I - 1 vial

9/25/99 COKE 9F9
3I 1 vial

9/11/99 COKE 9F9
3J 1 vial

B001561

A12 Clones

12/12/96 B8

1 vial:

2H6, 1H2, ~~5G4~~, 2F1, 6D12, 3A11, ~~2H11~~, 3H10

7E4, 6G5, ~~3B1~~, ~~6E2~~, 2C12, 1H1, 5G11, 5C7

~~8E6~~ (mold found in flask on 12/31/96), ~~2E3~~, ~~5A3~~

12/16/96 A1

1 vial:

8E4, 5F3, 8C11, 3G1, 5A11 (2 vials), 3C1, 3B5

8F1, 7D10, ~~4H9~~, 4C7, 4B11, ~~4G7~~, ~~8E3~~

6E6, 1H7, 3C1, 8D12, ~~3B1~~

12/18/96 B3

1 vial:

3H10, 6D12, 6E2, 2H11, 2F1, 1H2, 2C12, 3A11

2H6, 6G5, 5G4, 8E6 → (mold found in flask on 12/31/96), 7E4, 5G11, 3B1, ~~5A3~~

5C7, 1H1, 2E3

B001562

12/20/96 B4

1 vial:

8C11, 3G1, 3B1, 4B11, 4C7, 8E3

5A11, 3C1, 8F1, 4G7, 7D10, ~~4H9~~

3C1, 3B5, 5F3, 6E6, 2C11, 6F11

B1 1/7/97

2E4 (1) 2 vials

2E4 (2) 2 vials

1/21/97 6E2 ascites 5 vials ?

1/27/97 8E3 2 vials

2/13/97 5A11 ascites 1/2 vials

8E3 ascites 2 vials — ?

A12-Statine Clones

14/97	B1	6A6	2 vials
		1B7	2 vials
		3F2	2 vials
		3F12	2 vials
		1C9	2 vials
		5C7	2 vials
9/97		2B8	2 vials
		11E9	2 vials
		2H6	2 vials
0/97		4D3	2 vials
		6D9	2 vials
		4C7	2 vials
1/97		11E9 Ascites	2 vials
1/97		5C7 Ascites	3 vials
9/97		5C7 Ascites	1 vial
14/97		5C7 Ascites	2 vials

8/97 B3

6C3	2 vials	7F2	2 vials
2E2	2 vials	5C11	2 vials
7A12	2 vials	6E11	2 vials
8H9	2 vials	2E9	2 vials
9D6	2 vials	7B10	2 vials
6E10	2 vials	6A7	2 vials

3/98 C2

3D2	2 vials	3C5	2 vials
8B12	2 vials	2F12	2 vials
2D5	2 vials	4F5	2 vials

KW

5/4/99 F9
#21 2 vials
#12 2 vials
#34 2 vials

see p 101
for #34
#12

5/21/99 1B New Tank
KW 26 3 vials

1-C
#1 2 vials
#2 2 vials
#26 2 vials
} 5/99

1-D
#7 2 vials
#6 2 vials
} 5/99

1-I 6/10/99
#2 2 vials
#26 2 vials
#7 2 vials

1-D
KW 6 A? 4 vials 7/7/99

2A
KW 43 4 vials 7/26/99

2B KW 43 3 vials 8/99

A12 FF

B001569

5/25/00 4C 6E7 2 vials

6/00 4G 7D9 1 vial

4H 7D9 1 vial
6E6 PBS 4 vials

4I 2F12 2 vials
6E6 4 vials

8/24/00 4J 2C7 2 vials

4K 9C8 2 vials
5D3 2 vials
1C12 2 vials

4L 9B9 2 vials
5B1 2 vials
2B2 17 vials

8/28/00 4N 3F12 2 vials

5A
9/1/00 9E6 2 vials
9/5/00 8F1 2 vials

9/5/00 5B
4C7 2 vials
4G2 2 vials
6B6 2 vials

10/2/00 5F
6B4 x 2 vials
4E12 x 1 vial

10/2/00 5G
4E12 x 1 vial

11/27/00 5Q 9A11 x 2 vials

6C 8H12 x 2 vials
8H8 x 2 vials

6D 9B12 x 2 vials

11/28/00 6E 5F11 2 vials
6B8 2 vials
2B10 2 vials

6F 2G9 2 vials
9H10 2 vials
1F2 2 vials

6G 2B5 2 vials
4H9 2 vials
4D8 2 vials

12-5-00 6I 9A11 As x 3 vials

12-11-00 6I 11F9 x 2 vials
4A8 x 1 vial

6J 4A8 x 1 vial
1D3 x 2 vials
8H8 As x 3 vials

6K 3C5 x 2 vials
5G4 x 2 vials
1C9 x 2 vials

6L 10A10 x 2 vials
9B12 As x 2 vials

12-12-00 6L 5F11 As x 2 vials

6M 1F2 As x 2 vials
2A10 As x 2 vials

6N 7E7₂ x 2 vials
8A8₁ x 2 vials

6O 4F7 x 2 vials
5D4₃ x 2 vials
10H9₃ x 2 vials

6P 3H12 x 2 vials
9C1₁ x 2 vials
4G2₄ x 2 vials

12/14/00	7A	8H12 As × 2 vials
		1C9 As × 2 vials
12/20/00	7B	9H12 As 2 vials
		2G9 As 2 vials
		1H7₃ 2 vials
12/22/00	7C	10A10 As × 2 vials
		9B7 As × 1 vial
1/2/01	7D	1D3 × 2 vials
1/2/01	7E	9C1 As × 2 vials
		11F9 As × 2 vials
		5D4 As × 2 vials
	7F	3H12 As × 4 vials
		1D3 As × 2 vials
1/3/01	7G	4A8 As × 2 vials
	7H	4C2 As × 3 vials
1/8/01	7I	3C5 As × 1 vial
		1H7 As × 1 vial
1/9/01		1H7 As × 1 vial
1/17/01	7K	9H11 As × 3 vials
1/24/01	7L	8A8 As × 1 vial
	7L	8A8 As × 1 vial
	7L	7E7 As × 3 vials
9/27/01	9C	2G12 × 1 vial
	9E	2G12 × 1 vial
		7E11 × 2 vials
		1E9 × 2 vials
		1E8 × 1 vial
	9F	1E8 × 1 vial
10/14/01	9F	7E11 × 3 vials
	9G	1E9 As × 2 vials

Alz Sta II clones

10-12-00
5H
10A2 x 2 vials
15F10 x 17 vials

10-20-00
5I
10A2 As x 2 vials *put in liq. too early

10-21-00
HP 1A7 2 vials
8B3 2 vials

10-24-00

5H
10A2 As x 1 vial
5J
10A2 As x 1 vial

10/27/00
5K
6G10 2 vials
3D8 2 vials

10-31-00
5L
6G7 x 2 vials

11-2-00
5L
8B3 As x 2 vials
5M
1A7 As x 3 vials

5N 11/6/00
15F10 As x 3 vials

11/9/00
3D8 As x 3 vials

11-14-00
5,O
6G7 As x 2 vials

11-16-00
5P
2C10 As x 3 vials

11-17-00
5P
6G10 As x 1 vial

11/27/00
5Q
6C5 x 2 vials
1C7 x 2 vials

6C
2C10 x 2 vials

12/14/00
7A 1C7 As x 2 vials

2-16-01
7M 6C5 As x 3 vials

8/28/01
9A 6E2 x 2 vials
9G2 x 2 vials

9B 7B1 x 2 vials

9/12/01
9C 6E2 As 4 vials
7B1 As 1 vial

9D 7B1 As 2 vials

A|z FX clones

2/27/01 7N
A|z FX 3C11 x 3 vials

3/5/01 7O
5D10 x 2 vials

3/12/01 8B
3C11 AS 4 vials

3/31/01 8E 5D10 AS. 2 vials

H12 KL clones

3/5/01 70
5D11 x 2 vials

3/2/01 8A
8D2 2 vials

8B
4A5 2 vials

8C
4A5 2 vials

3/27/01 4A5 As x 4 vials

3/27/01 8D
6F9 As x 3 vials
5D11 As x 3 vials

3/31/01 8F 8D2 As 3 vials

4/12/01 8H 14D7 2 vials
3H1 2 vials
8I 13B11 2 vials

6/21/01 8K 15F12 x 2 vials

6/29/01 8L 3H1 As x 2 vials

7/9/01 8M 15F12 As x 3 vials
13B11 As x 2 vials

7/31/01 8P 4H4 x 2 vials

8Q 4H4 x 1 vial

Alz X F

6/12/01
8J 5G5 2 vials

6/21/01
8K 2C7 2 vials

6/28/01
8L 5G5 As x 2 vials

7/31/01 8P 2C7 As x 2 vials

Alz KL super

7/9/01	8N	- 4H4 x 2 vials
		5C8 x 2 vials
		8C9 x 2 vials
7/31/01	8O	5C8 As x 3 vials
		8C9 As x 3 vials
8/3/01	8R	4H7 2 vials
	8S	3E9 2 vials
		5B4 2 vials
		3F9 2 vials
10/15/01	9G	3E9 As. 2 vials
10/22/01	9H	3F9 As 3 vials
12/5/01	9M	8C3 2 vials
		1E3 2 vials

EOC-2 microglial

~~7/31/01 8P EOC 2 - ~~2~~ 0+ vials~~

9/6/01 9B EOC2 - 1 vial

~~12/19/01 10A - 1 vial~~

5/7/02 10N 3 vials

10O 2 vials

5/28/02 10P ~~4 vials~~ ~~3 vials~~ 2 vials

TMB 1

8/1/01 8A 2 vials

8/9/01 9A 2 vials

9/17/01 9D ~~4~~ 3 vials TMB As.

12/5/01 9D 2 vials

Alz II clones

2/11/02	10H	6E9	3 vials
	10J	6E9 Asc.	2 vials

7/13/02 KLH - Hep4 clones

10K	10A12	1 vial
1F	10A10b	3 vials
1K	5E3	3 vials
1L	8C4	2 vials
1O	8C4	1 vial
2B	1B1a	2 vials
2A	6D2	1 vial
2H	9D9	1 vial
2I	8G2b	1 vial

KL3 Clones

3/13/02	10K	4D8b	3 vials
3/28/02	10L	3H2	4 vials
4/11/02	10W	3H2 Asc.	2 vials

MN

5/28/02 10 0 3 vials

<u>HN</u>

5/28/02 10 P 1 vial

7/13/02 XH - II clones

1 R	4 C1 2	1 vial
2 D	1 C10	1 vial

7/3/02 P3C - Hep clones

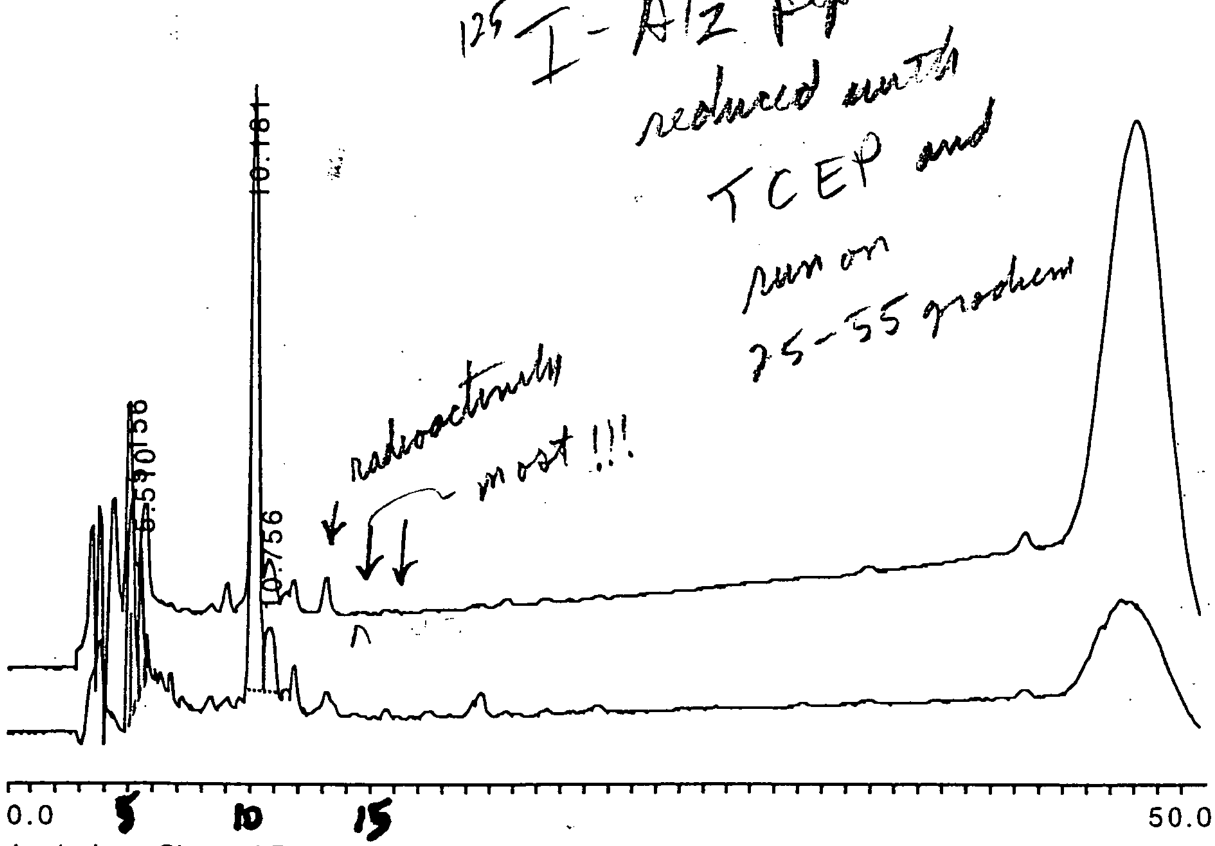
2F	8B4	2 vials
2G	8B4	1 vial

B001584

Date: Sun, Dec 15, 1996 10:42 AM
Data: 25-55%-15DEC96-001

Processing File:
Method: 25-55%
Sampling Int: 0.1 Seconds

Data:



Analysis: Channel B

Peak No.	Time	Type	Height(µV)	Area(µV-sec)	Area%
	5.020	N1	2404	9964	6.348
1	5.156	N2	2391	17910	11.410
2	5.510	N3	1541	10094	6.430
	5.683	N4	452	3135	1.997
3	10.181	N1	6798	95832	61.055
4	10.756	N2	732	20024	12.757
Total Area				156959	99.997

B001585

12/30/96

Alz clones

PEG Assay

use for Ascites 6E2

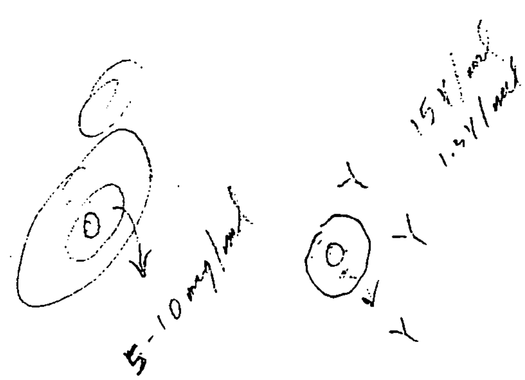
8E3

green

1	1.00	7663 2H6 ✓	45
2	1.00	9338 2E3 ✓	53
3	1.00	8624 5A3	50
4	1.00	7641 2C12 ✓	59
5	1.00	*15727 5G4 ✓	52
6	1.00	6125 3B1	55
7	1.00	6774 3H10	46
8	1.00	7920 6D12 ✓	56
9	1.00	*30834 6E2 ✓	39
10	1.00	*13231 8E6 ✓	42
11	1.00	9330 5C7 ✓	58
12	1.00	7597 3A11	53
13	1.00	7841 6G5	43
14	1.00	7924 1H1	52
15	1.00	*11285 2F1	62
16	1.00	*11442 1H2 ✓	67
17	1.00	5790 5G11 ✓	41
18	1.00	4889 2H11 ✓	47
19	1.00	6197 1E11	48
20	1.00	8772 Hy	52

Violet

1	1.00	*17106 3B1 ✓	45
2	1.00	*10561 4B11 ✓	56
3	1.00	9267 4C7 ✓	56
4	1.00	*14188 8D12 ✓	57
5	1.00	9069 6F11 ✓	53
6	1.00	9165 2C11	42
7	1.00	5469 8F1	59
8	1.00	*15881 4G7 ✓	55
9	1.00	6294 5A11 ✓	54
10	1.00	9312 8C11	50
11	1.00	9153 6E6 ✓	55
12	1.00	*18408 8E3 ✓	58
13	1.00	8369 3C1	65
14	1.00	6116 4H9 ✓	47
15	1.00	5387 8E4	48
16	1.00	6467 3B5	44
17	1.00	6729 7D10	45
18	1.00	6698 5A11	46
19	1.00	9622 3C1 ✓	50
~~20~~	~~1.00~~	~~9254 1H7~~	~~55~~
21	1.00	5349 5F3	51
22	1.00	7369 3G1	51
23	1.00	11219 Hy	40
24	1.00	51	46



B001586

1	1.00	6060	5F3	65
2	1.00	5901	8C11	44
3	1.00	6733	8F1	59
4	1.00	5561	5A11	64
5	1.00	6679	6E6	61
6	1.00	7800	3B5	66
7	1.00	7054	3G1	47
8	1.00	6956	8E4	58
9	1.00	6690	1H7	47
10	1.00	7367	3B1	49
11	1.00	9316	4H9	53
12	1.00	8333	5A11	53
13	1.00	7730	8D12	59
14	1.00	6752	7D10	62
15	1.00	9646	3C1(1)	49
16	1.00	6097	4C7	42
17	1.00	5414	4G7 ✓	63
18	1.00	8556	4D11	48
19	1.00	*20587	8E3	54
20	1.00	7496	3C1(2)	42
21	1.00	10791	H4	47
22	1.00	50		44

B001587

PEG assay
12/31/96

1	1.00	6690	6E2	51
2	1.00	8636	[illegible] 1:10	53
3	1.00	11097	[illegible]	52
4	1.00	9607	[illegible] 1:10	39
5	1.00	16662	Hy	51
6	.02	2		2

B001588

PEG Assay
12/31/96

A/2 Vials

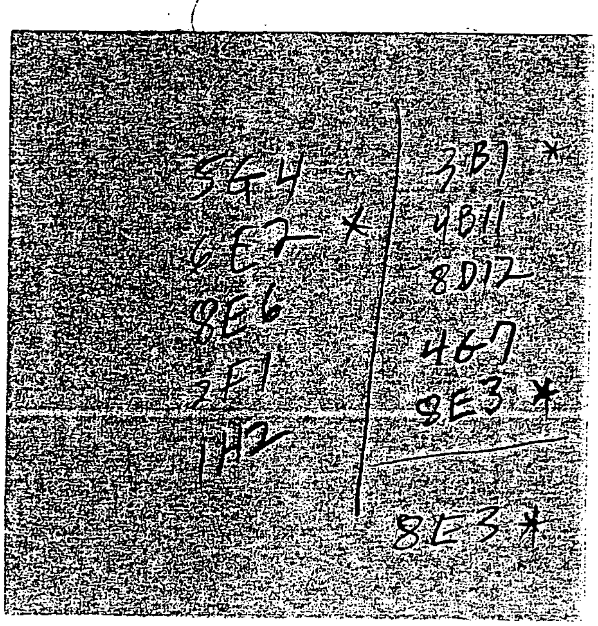
1	1.00	5609	6E2	49
2	1.00	52262	6E2old	50
3	1.00	13863	8E3	62
4	1.00	34807	8E3old	41
5	1.00	15580	Hg(H)	46
6	1.00	7145	Hg(B)	51
7	1.00	65		47

B001589

PEG assay
12/30/96

1	1.00	11982	5G4	44
2	1.00	15938	6E2	47
3	1.00	9379	6E2 1:10	48
4	1.00	8814	7E4	63
5	1.00	12078	3B1	43
6	1.00	3353	3B1 1:10	52
7	1.00	10789	8E3	41
8	1.00	3249	8E3 1:10	64
9	1.00	3171	Hy old	51
10	1.00	2903	Hy new	38

A12 clones



^{125}I A121-43

Fractions from HPLC run

1λ assayed from 200λ of H_2O added to each tube

1	1.00	449777	1	52
2	1.00	41333	6	73
3	1.00	69325	7	46
4	1.00	108490	8 →	54
5	1.00	97956	9	35
6	1.00	42806	10	38
7	1.00	343251		67
8	1.00	60		57
9	1.00	50		42
10	1.00	54		54

1×10^5 cpm/1λ

epindorf tube with 50% AcNitrile

on gel run

1	1λ	= ~ 500,000 cpm	
6	12λ	"	
7	8λ	"	
8	5λ	"	
9	6λ	"	
10	~~12~~λ	"	
x	1.5	"	

use 50λ of # 8 = 5×10^6 cpm

1 50λ ~~+ 5λ 10x PBS~~
+ 5λ 10xPBS + 150λ PBS = 200λ for i.v. injection

in glass tube
check syringe after injection

B001591

^{125}I-ALZ 1-43
tube # 7
1λ

1	1.00	3676	Hy	43
2	1.00	3308	3B5	58
3	1.00	11885	6E6	70
4	1.00	11922	8E4	44 — 12/13/96
5	1.00	3415	3B1	50
6	1.00	2904	HH9	54
7	1.00	3015	8E4	45 — 12/20/96
8	1.00	10290	6D12	54
9	1.00	11619	6E2	57
10	1.00	5503	5C7	37
11	1.00	27933	supernatant	62 ← supernatant from Hy #1 PEG
12	.02	130		1

1	1.00	445413	58	
2	1.00	33745	49	
3	1.00	61399	48	
4	1.00	97984	51	
5	1.00	85259	48	
6	1.00	31518	50	
7	1.00	279022	50	
8	1.00	60	42	
9	1.00	59	37	
10	1.00	64	53	
1	1.00	7178	47	// 1.6×10^6 cpm expected 5×10^6
1	.62	1015827	42	— 50λ of #8 diluted into 150λ sterile PBS
2	1.00	55134	53	← pippette tip
3	.51	33	24	

PEG 4/2/96

1 PBS
2 PBS
3 Hy
4 Hy
5 5H11
6 2E3
7 5G11
8 2H11
9 1H2

1	1.00	25627	PCS
2	1.00	24159	
3	1.00	5163	Hy
4	1.00	4264	Hy
5	1.00	47871	5A11
6	1.00	15360	2E3
7	1.00	7682	5G11
8	1.00	8818	2H11
9	1.00	5891	1H2
10	1.00	138	

2/27/99

7D3/5A11 hybrid @ ~ 10 mg/ml

10 ug/ul

use 8λ hybrid (80 ug) 80 ug

2λ carbonate buffer (0.5 M pH 9)

use 0.07 mg Fitc / mg Hybrid

0.07 ug / ug Hybrid

X 80 = 5.6 ug

5.6 mg/ml = 5.6 ug/ul ... 1 ul

make

0.45 mg

$\frac{5.6}{1} = \frac{0.45}{x} = 80λ$ use 1

160λ use 2

B001596

9/12/97 ELISA

* dilute Brain Extract 1:50 w/ 1M TRIS pH8.0

			w/5A11	w/out 5A11
1	human serum 1:10	in PBS		+
2	human serum 1:100	in PBS		
3	human serum 1:1000	in PBS		
4	human serum 1:10,000	in PBS	+	
5	BE 1:10	in PBS	+	
6	BE 1:100	in PBS	+	
7	BE 1:1000	in PBS	+	
8	BE 1:10000	in PBS	+	
9	PBS		+	
10	PBS		+	
11	PBS		+	
12	PBS		+	

* test 2 rows, one w/ 5A11 & one w/out 5A11

Rinse w/ PBS

Block w/ BSA

Add 5A11 Ab

Add anti Fc

top row w/ 5A11

5A11 @ 3.7×10^{-4} M

dilute 1/1000 = 3.7×10^{-7} M
in PBS

use 100 γ/well

B001597

ELISA 10/~~28~~29/97

			Ab ϕ sta	1-HO
1	Hy			
2	Hy			
3	Blue	10λ	–	–
4	Blue	1λ	–	–
5	Blue	.1λ	–	–
6	Red	10λ	(not that dark) +	–
7	Red	1λ	+/–	–
8	Red	.1λ	+/--	–

B001598

FLISA 10/17/97

Alz 1-43 plate

Hy
serum
5A11
3D9¹ 20λ
3D9¹ 2λ
3D9² 20λ
3D9² 2λ
3D9³ 20λ
3D9³ 2λ

anti Fc 1:10,000

Results
Serum came up slightly (+)

*D6B3 ⊖ control

* Hold off on T-gel Ab's unless everything is working OK

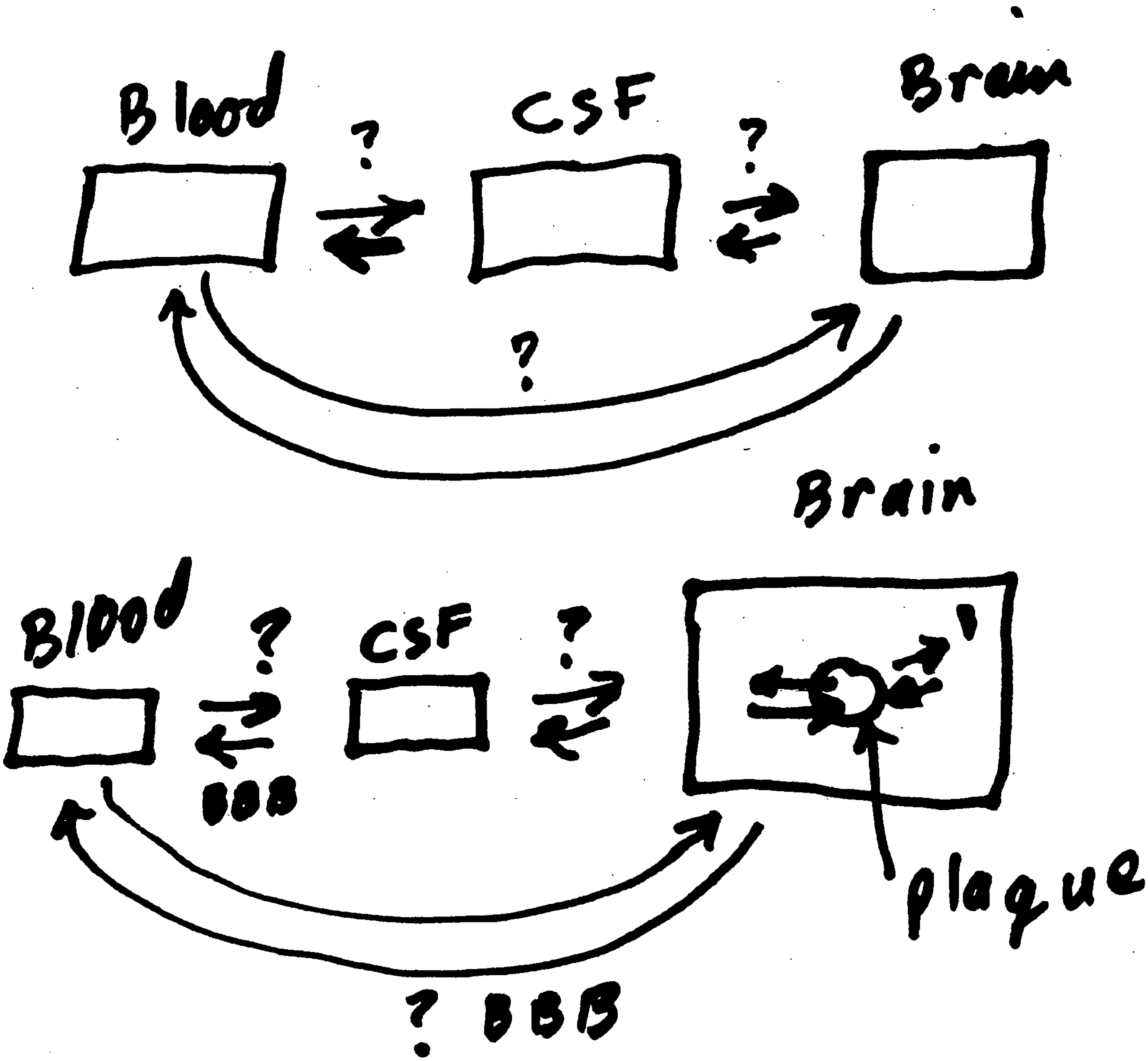
Ab	Antigens		
6E11	HIV PS II	V3 loop	GPG
3H3	"	"	"
7B1 Tgel	"	"	"
3E4 Tgel	"	"	"
9E1	"	"	"
4D2	"	"	"
3H8	"	"	"
7B3	"	"	"
5C7	Alz Sta	Alz C terminal	Alz 1-43
11E9	Alz Sta	Alz C terminal	Alz 1-43
8E3 Tgel	Alz PS	Alz ~~15~~ 17 mer	Alz 1-40 ~~Alz 2-43~~
6E2 T-gel	Alz PS	Alz ~~15~~ 17 mer	" ~~Alz 1-43~~
5A11	Alz PS	Alz ~~15~~ 17 mer	" ~~Alz 1-43~~

* dissolve peptide (not too much) in PBS ~100 λ

spot ~ 1 λ

use a very small amount of peptide on end of pipet tip if need to increase volume to 200-300 λ d.o. ∞

B001600



B001601

1 CEM
2 CEM + Fitc 7D3/5A11 ← use 5γ
3 ECV
4 ECV + Fitc 7D3/5A11 – use 5γ
5 CEM + ~~7D3/5A11 +~~ 1-43 (5) → 1γ
6 CEM + 7D3/5A11 + 1-43 (5)
7 CEM + 1-43 (11) → 10γ
8 CEM + 7D3/5A11 1-43 (11)
9 CEM + 1-40 (4)
10 CEM + 7D3/5A11 + 1-40 (4)
11 CEM + ~~7D3/5A11~~ + 1-40 (5)
12 CEM + 7D3/5A11 + 1-40 (5)

7D3/5A11
use 0.3γ

CEM in 1.2 ml PBS

~~use 1 ml / tube~~ 100γ / tube

ECV in 0.5 ml use 100γ / tube

for cells on coverslip
use 7D3/5A11 use 2γ
use 0.3 + 10γ of 1-40 (5)

B001603

2/27/99

7D3/5A11 hybrid @ ~ 10 mg/ml

use 8λ hybrid (80 ug) 10 ug/ul
80 ug

2λ carbonate
buffer
(0.5M pH9)

use 0.07 mg Fitc / mg Hybrid
0.07 ug / ug Hybrid
X 80 = 5.6 ug

5.6 mg/ml = 5.6 ug/ul ... 1 ul make

0.45 mg

$\frac{5.6}{1} = \frac{0.45}{x} = 80\lambda$ use 1
1 80λ use 2

B001604

~ 5 hours after i.v. injection   11/29/96   Friday

	wt	cpm - Bkg ~60	cpm/gm	B/B - Bkg
blood	23.8 mg	1,976	83.0 X 10^3	1.0
brain	387.5 mg	1,094	2.8 X 10^3	0.034
spleen	109.6 mg	8,840	80.7 X 10^3	0.97
kidney	171.2 mg	14,890	87.0 X 10^3	1.05
heart	113.3 mg	3,150	27.8 X 10^3	0.33
liver	376.4 mg	71,872	190.9 X 10^3	2.3
muscle	101.7 mg	1,544	15.2 X 10^3	0.18

37361
36228
.1133

37854
36142
.1712

37311
36315
.1096

40079
36315
.3764

37177
36160
1017

3.6592
3.6354
238

4.0180
3.6305
.3875

brain/blood

$2.8 \times 10^3 / 83 \times 10^3 =$

3.4×10^{-2}

3.4 X 100%

~ 5 hours after
1.V. injection of immunized mouse
(green) AL2 phenyl statine

		cpm	bkg (60) cpm / gm	(- Bkg) B/B
blood	0.1236 gm	2,203	17.8 $\times 10^3$	1.0
brain	.4385	330 - 57	0.75 $\times 10^3$	.042 (0.62 $\times 10^3$
spleen	.1660	2,402	14.4 $\times 10^3$	0.81
kidney	.2550	5,442	21.3 $\times 10^3$	1.2
heart	.1685	1,398	8.3 $\times 10^3$	0.47
liver	.3757	10,012	26.6 $\times 10^3$	1.49
muscle	.1623	456	2.8 $\times 10^3$	0.16

blood
3.7764
3.6528
.1236

brain
39906
35521
.4385

spleen 38053
36393
1660

liver 4.0204
36447
.3757

$$B/B \quad \frac{0.75 \times 10^3}{17.8 \times 10^3} = 4.2 \times 10^{-2}$$
42 × 1000

kidney 38906
36356
.2550

$\frac{0.62}{17.8} = 35 \times 1000$

heart 37427
35737

muscle
38018
36395
.1623

B001606

STAT FAX 2100 :S SN· 1112 09/18/99 13:27:43
ABSORBANCE MODE 12 PAGE 1 09/18/99 13:27:46
LOT NUMBER:______ EXP. DATE:______ USER:______
WAVELENGTHS=450NM 630NM

	1	2	3	4	5	6	7	8	9	10	11	12
1A	0.364	2.088	2.326	1.194	0.380	0.886	0.799	0.950	0.659	0.769	0.311	0.409
1B	0.987	0.621	0.426	0.383	0.743	0.264	0.715	0.442	0.465	0.663	0.772	0.226
1C	0.223	0.602	1.194	0.835	0.593	0.322	0.448	0.342	0.886	0.885	0.754	2.576
1D	3.174	3.275	3.280	3.336	2.819	3.223	1.681	2.965	3.317	3.280	3.115	1.986
1E	2.864	0.724	0.572	0.000	0.000	0.000	0.000	-0.000	0.000	-0.000	0.001	-0.000

END OF RUN

assume 20 moles M / c BSA

↑ mW = 67,000

$\frac{.65 \times 10^{-6}}{2\,mg = 3 \times 10^{-8}\,moles} = 22$

~~5 mg~~ 5 mg = 7.5×10^{-8} moles $\times 20 = 1.5 \times 10^{-6}$ m

5 x excess = 7.5×10^{-6} moles

1 x excess = 1.5×10^{-6} moles = $\boxed{1X = 4.6\,mg}$

ALZ 1-25 cys MW = 3035 = 22.6 mg

~~have ~ 13 mg~~

5 mg immunize monkey with 100 ug / injection = 50 injections

ALZ 22-42 cys = 2102 = 15.8 mg $\boxed{1X = 3.2\,mg}$

we have ~ 6 mg = ~ 2X excess ↑ try 1X excess

final volume

2 ml ½ H_2O ½ pH 8 0.1M PO_4

ELISA Results
from A12 Sta Clones

B001609

9/4/[illegible]
Testing H2 sera clones
from 11/9/97 fusion

	1	2	3	4	5	6	7	8	9	10	11	12
A	H4	9F2	9B6	9F1	9F11	H2 SFC serum	Mouse serum	1C3	1B11	1D10	1G7	1H2
B	2A8	2C8	2C10	2E2	2F5	3A2	3B2	3F2	3F7	4F3	4F6	4H6
C												
D												
E												
F												
G												
H												

[illegible] plate

Used 50 uL from well / 50 uL H4
Used 1 uL of sera / 100 uL H4
Repeating 9F2, 9F6, 9F1, + 9F11 [illegible]
[illegible])

Results
± H2 SFC serum
± 9F2
± 9F1
± 2B5

Testing ± Sca clones from 1/9/97 fusion

[illegible]

	1	2	3	4	5	6	7	8	9	10	11	12
A	Ag	[illegible]	[illegible]	5A7	6B10	7C5	5C11	5D1	5D11	6E6	6E7	6F1
B	6F5	6H4	6H4	6C6	6E1	6E9	6F1	6H7	8B10	8F8	10H2	11H1
C												
D												
E												
F												
G												
H												

Used media from wells

Used 100 of sera

Results:

± to Sca binding

* no ± clones

	1	2	3	4	5	6	7	8	9	10	11	12
A	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
B	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C												
D												
E												
F												
G												
H												

[illegible]

	1	2	3	4	5	6	7	8	9	10	11	12
A	[illegible]	[illegible]	[illegible]	1H1	~~[illegible]~~	~~[illegible]~~	~~1C10~~	1E2	1E3	1F12	1H11	3A1
B	3B10	3F4	3H6	3A11	3B1	3A3	3D8	3E2	3E7	3F10	3G7	3H6
C												
D												
E												
F												
G												
H												

Results:

(+) HE 5th serum

+/- 1H1 ? (slightly yellow)

+/- 3H6 ? (slightly yellow)

B001613

	1	2	3	4	5	6	7	8	9	10	11	12
A	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]		
B												
C												
D												
E												
F												
G												
H												

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

[illegible]

[illegible]

	1	2	3	4	5	6	7	8	9	10	11	12
A	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
B	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
C	[illegible]	8A11	8A12	[illegible]	8D10	8D11	8D12	[illegible]	8C2	[illegible]	[illegible]	[illegible]
D	8E3	[illegible]	8E6	8F9	[illegible]	[illegible]	[illegible]	[illegible]	9A1	[illegible]	[illegible]	[illegible]
E	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
F	9F7	9G3	9G4	9G11	9H2	[illegible]	10A7	10B2	10B4	10B11	10C6	10D2
G	10D6	10E1	10E7	10F6	10F7	10F9	10F11	10G5	10G9	[illegible]	10H3	10H5
H	10H6	10H11	11A3	11A5	11A7	11A11	11B5	11B6	11B7	11B8	11C8	11D6

Results.

[illegible]

[illegible]

	1	2	3	4	5	6	7	8	9	10	11	12
A	Hi4	3P8	3H6	3F2'	H03	5C7	W46	908	W09			
B	Hi4	3P8	3H6	3F2'	H03	5C7	W46	908	W09			
C												
D	Hi4	3P8	3H6	3F2'	H03	5C7	W46	908	W09			
E	Hi4	3P8	3H6	3F2'	H03	5C7	W46	908	W09			
F												
G												
H												

Doing a comparison between the [illegible] plate + the β amyloid 1-42 plate using 100 uL + 10 uL samples [illegible]

[illegible]

100 uL [illegible]

3P8 +-
3H6 +
3F2' ++
H03 +
5C7 +++
W46 ++
[illegible] +
[illegible] +-

10 uL [illegible]

5C7 ++
W46 +
908 +

100 uL 1-42

[illegible] +
[illegible] +
[illegible] +
[illegible]
[illegible]

10 uL 1-42

5C7 [illegible]
W46 [illegible]
[illegible]

B001617

1/27/97

	1	2	3	4	5	6	7	8	9	10	11	12
A	1H4	+/- 2B8	+ 2H6	++ 3F2'	+/- 4D3	++ 5C7	++ 6H6	+/- 9D8	+/- 6D9'			
B												
C												
D												
E												
F												
G												
H												

Alz Sta Dimer plate

Testing Alz Sta clones, used 100 uL from limbro wells

Results:

All are +

3F2'
2H6
5C7
6H6
} These are the darkest

The Alz Sta plate gave darker results
This assay appeared to be much lighter.

#12 Sequence
1/21/97

[illegible]

	1	2	3	4	5	6	7	8	9	10	11	12
A	4H4	[illegible]	[illegible]	3H10	3H12	3A2	3B7	3C2	3C4	3C7	5C12	3C7
B	3C12	3E6	3E8	3E11	3E12	3F9	3F10	3G9	3G10	3G12	3H1	3H3
C	3H4	3H5	3H6	3C1	3C4	3D9 (+)	3D10	3C11	3E10	3F1	3F4	3F9
D	3F11	3F12 (+)	3G3	3G4	3G5	3G6	3G10	3H1	3H12	4H3	4A4	4A10
E	4A10	4C10	4C11	4C4	4E3	4E5	4E6	4F4	4F7	4F9	4G3	4G4
F	4G12	4H1	4H3	4H4	4H5	4H9	5A6	5A10	5A11	5B1	5C6	5D5
G	5E7	5E12	5F6	5H5	6A1	6A12	6B2	6B8	6C4	6C7	6C9	6E12
H	6F11	6G3	6H6	1E7	1E12	1B6	1C7	1D10	1C7	1C9	1C9 (+)	1C12

Results:

1C9 ±
3D9 ±
3F12 ±
1H3 (±)

A/2 Sta clones
1/28/97

A/2 Sta plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	HY	A/2 Sta serum	linker sera	7A1	7A11	7A12	7C7 (+)	7D1	7E2	7E3	7E4	7F10
B	7F11	7G11	7H7	7H8	8B3	8C3	8C7	8C10	8C11	8D1	8E12	8F1
C	8F2	8G4	8G12	8H7	8H9	8H10	9A4	9A6	9B11	9C12	9D10	9E1
D	9E2	9E3	9E9	9E10	9F12	9G1	9G2	9H4	9H5	9H10	9H12	10A1
E	10A6	10A9	10A10	10A11	10C9	10D12	10E2	10E10	10F1	10F2	10F12	10G2
F	10G6	10H8	10H12	11B4	11C2	11C3	11C4	11C11	11C12	11D4	11E5	11E9 (+)
G												
H												

Results:
7C7 (+)
11E9 (+)

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

B001621

Testing A12 Sta clones

	1	2	3	4	5	6	7	8	9	10	11	12
A	1H4	2B8	2H6	3F2	4D3	5C7	6A6	7C7	11E9	1E4		
B				+		+	+	+				
C												
D												
E												
F												
G												
H												

t12
1-40
plate

Used 100 ul from lumaro wells

Results:

3F2
5C7
6A6
7C7
} (+)

ALZ 1-40

STAT FAX 2100 :S SN 1112 01/31/97 17:21:07
ABSORBANCE MODE 12 PAGE 1 01/31/97 17:21:24
LOT NUMBER:________ EXP. DATE:________ USER:________
WAVELENGTHS=450NM 630NM

	1	2	3	4	5	6	7	8	9	10	11	12
1A	-0.028	0.001	0.001	0.001	0.001	0.000	0.001	0.001	0.001	0.001	0.001	0.00[illegible]

END OF RUN

	1	2	3	4	5	6	7	8	9	10	11	12
2A	0.084	0.131	0.257	0.685	0.264	0.714	0.664	0.783	0.120	0.109	-0.006	-0.008
	Hy	2B8	2H6	3F2	4D3	5C7	6A6	7C7	11E9	1E4		
2B	0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.000	0.000

B001623

Testing A12 Sta clones

	1	2	3	4	5	6	7	8	9	10	11	12	
A									Ny	2B8	2H6	3F2	1-H3
B	+ 4D3	+ 5C7	+ 6H6	9D8	6D9	1C9	1B7	3F12	+ 7C7	11E9	1D12	1E4	
C													
D									Ny	2B8	2H6	3F2	A12 Sta
E	+ 4D3	+ 5C7	+ 6H6	9D8	6D9	1C9	1B7	3F12	+ 7C7	+ 11E9	1D12	1E4	
F													
G													
H													

Doing a comparison assay between A12 Sta plate & pamyloid 1-H3 plate

Used 100 uL from limbro wells.

Results

A12 Sta	1-H3
+ 4D3	+ 4D3
+ 5C7	+ 5C7 (dark)
+ 6H6	+ 6H6
+ 7C7	+ 7C7 (dark)
+ 11E9 (very dark**)	

A12 Sta Clones

STAT FAX 2100 :S SN 1112 01/31/97 13:14:39
ABSORBANCE MODE 12 PAGE 1 01/31/97 13:14:58
LOT NUMBER:__________ EXP. DATE:__________ USER:__________
WAVELENGTHS=450NM 630NM

	1	2	3	4	5	6	7	8	9	10	11	12	
1A	0.000	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.088	0.316	0.933	0.617	
									4G9	2B8	2H6	3F2'	A12
1B	0.814	1.988	1.479	0.186	0.14[illegible]	0.182	0.188	0.264	2.556	3.423	0.358	0.630	Sta
	4D3	5C7	6A6	9D8	4D9'	1C9	1B7	3F12	7C7	11E9	1D12	1E4	plate
1C	0.006	0.009	0.002	0.008	0.006	0.006	0.009	0.009	0.004	0.002	0.002	0.002	
1D	0.000	0.000	0.000	0.000	0.000	0.[illegible]	-0.000	0.000	0.202	0.146	0.754	0.809	
									4G9	2B8	2H6	3F2'	1-A3
1E	0.629	2.150	1.201	0.124	0.112	0.3[illegible]	0.186	0.436	2.217	0.127	0.493	0.160	
	4D3	5C7	6A6	9D8	6D9'	1C9	1B7	3F12	7C7	11E9	1D12	1E4	plate
1F	0.001	0.000	0.000	0.001	0.001	0.001	0.001	0.000	0.000	0.000	0.001	0.001	

END OF RUN .

.217

B001625

Testing clone that was separated.

Atz sta plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Hy	Atzsta serum	marker	3D9'	3D9²							
B												
C												
D												
E												
F												
G												
H												

Result:

3D9' ⊕

STAT FAX 2100 :S SN 1112 02/12/97 15:08:55

ABSORBANCE MODE 12 PAGE 1 02/12/97 15:09:04

LOT NUMBER:______________EXP. DATE:________________USER:________________

WAVELENGTHS=450NM 630NM

	1	2	3	4	5	6	7	8	9	10	11	12
1A	0.059	0.115	3.439	2.841	1.424	3.242	2.636	1.183	1.830	2.363	0.979	2.724
	Hy	4B1										
1B	3.152	0.883	0.001	0.001	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.001
1C	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001
1D	0.073	0.101	1.645	1.237	0.771	1.698	1.284	0.639	0.311	0.100	0.121	1.210
1E	0.788	0.326	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001
1F	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000
1G	0.000	0.000	0.000	-0.000	0.000	0.000	0.000	0.000	0.000	-0.000	0.001	0.000
1H	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

END OF RUN

STAT FAX 2100 :S SN 1112 01/07/97 17:37:57

ABSORBANCE MODE 12 PAGE 1 01/07/97 17:38:04

LOT NUMBER:________ EXP. DATE:________ USER:________

WAVELENGTHS=450NM 630NM

	1	2	3	4	5	6	7	8	9	10	11	12
1A	0.445	0.843	0.627	1.765	0.381	0.484	-0.001	-0.001	-0.001	-0.001	-0.000	0.000
	Black	Blue	Yellow	Red	Green	cont.						
1B	-0.000	-0.001	0.000	-0.000	-0.000	-0.000	-0.001	-0.000	-0.001	-0.001	-0.001	-0.000
1C	1.809	1.949	1.251	1.793	1.789	0.384	0.000	0.000	0.001	0.000	0.000	0.000
1D	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000

¿END¿OF¿RUN¿¿

B001628

3/20/97

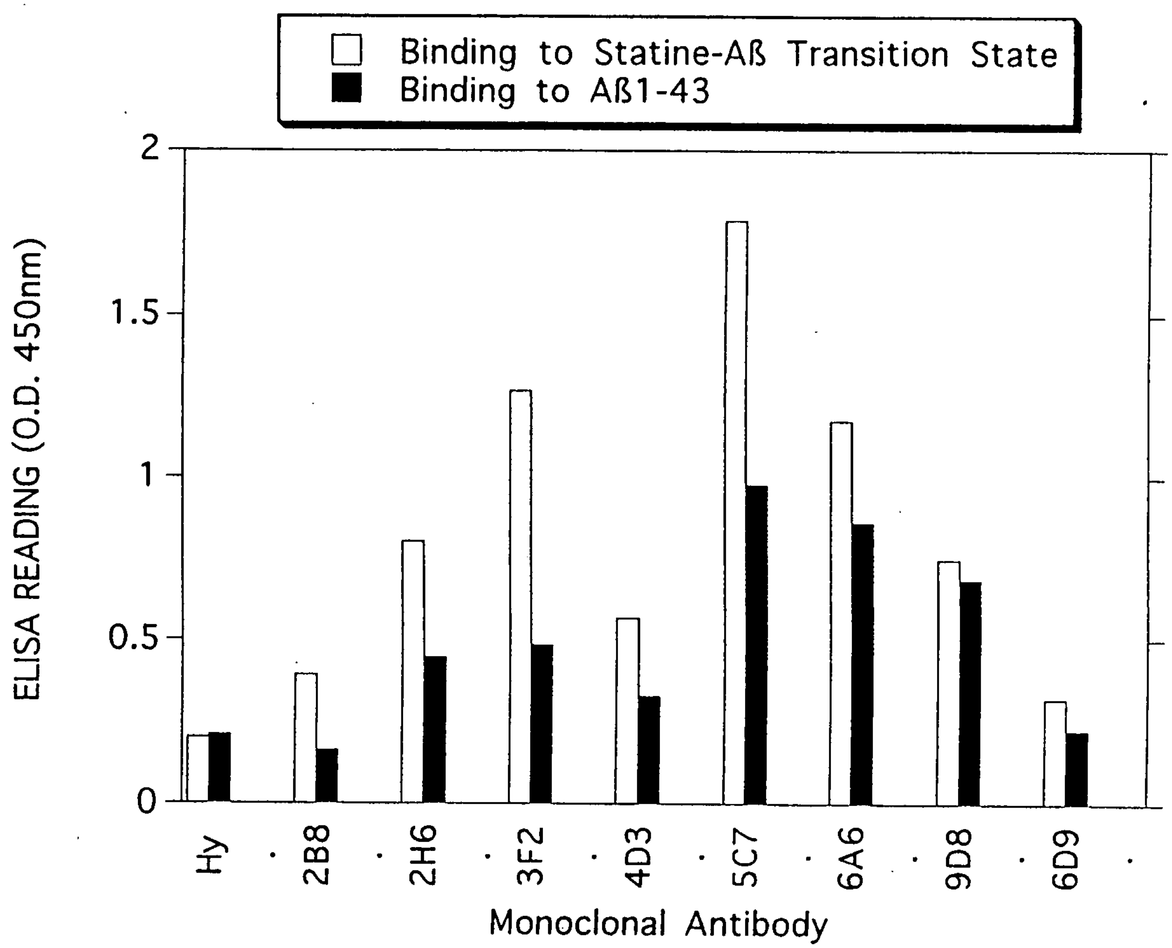
Alz Sta plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Hy	Alz Sta serum	11E9 sup +	11E9	5C7							
B												
C												
D												
E												
F												
G												
H												

Testing 11E9, + 5C7 (Alz Sta clones) to see if still producing Antibody.

Results:
~~X~~ only Alz Sta serum showed to be positive

B001629



Binding to Statine-Aß Transition State
Binding to Aß1-43
ELISA READING (O.D. 450nm)
0
0.5
1
1.5
2
Hy
2B8
2H6
3F2
4D3
5C7
6A6
9D8
6D9
Monoclonal Antibody

B001630

Ab Sta clones

STAT FAX 2100 :5 SN 1112 01/13/97 16:50:16
ABSORBANCE MODE 12 **PAGE 1** 01/13/97 16:51:31
LOT NUMBER:________ EXP. DATE:________ USER:________
WAVELENGTHS=450NM 630NM

	1	2	3	4	5	6	7	8	9	10	11	12
1A	0.000	0.000	0.023	1.951	0.023	0.530	1.296	0.031	0.019	1.336	0.423	0.745
		*	Hy	5G4	6D12	3B1	8E6	8E11(2)	8E11(1)	6F11(2)	4G7	5H11(2)
1B	1.743	1.827	1.454	1.324	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.001
	3B5	8E3	6E6	6F11(1)								
1C	0.170	0.023	0.036	0.026	0.029	0.024	0.960	0.022	0.030	0.051	0.834 *	0.886
	Hy	6D12	3B1	8E6	8E4(2)	8E11(1)	6F11(2)	4G7	3B5	8E3	6E6	6F11(1)
1D	0.234	1.078	-0.001	0.000	-0.000	-0.000	-0.001	-0.000	-0.000	-0.000	0.000	-0.000
	5A11(2)	6E2										
1E	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.001	0.001	0.001	0.000	0.001

END OF RUN

injected 6F11 (1)
and 8E3

B001631

A12 Stackones

ABSORBANCE MODE 12 PAGE 1 01/27/97 11:57:13

LOT NUMBER:______ EXP. DATE:______ USER:______

WAVELENGTHS=450NM 630NM

		1	2	3	4	5	6	7	8	9	10	11	12	
100uL	1A	0.201	0.393	0.802	1.268	0.566	1.785	1.178	0.748	0.321	0.000	0.001	0.001	A12 Stock plate
		Hy	2B8	2H6	3F2'	4D3	5C7	6A6	9D8	6D9'				
10uL	1B	0.193	0.129	0.156	0.226	0.167	0.660	0.371	0.262	0.127	0.001	0.001	0.001	
	1C	0.000	0.000	-0.001	0.000	0.000	0.000	-0.000	-0.000	0.000	0.000	0.000	0.000	
100uL	1D	0.209	0.163	0.446	0.482	0.327	0.976	0.862	0.685	0.223	0.000	-0.001	-0.000	1-H3 plate
		Hy	2B8	2H6	3F2'	4D3	5C7	6A6	9D8	6D9'				
10uL	1E	0.087	0.058	0.143	0.143	0.126	0.310	0.304	0.327	0.241	0.000	0.000	0.000	
	1F	0.000	0.000	0.000	0.000	-0.000	-0.000	-0.000	-0.000	0.000	-0.000	0.000	0.000	

END OF RUN

B001632

1/23/97

STAT FAX 2100 :S SN 1112 01/23/97 14:26:46

ABSORBANCE MODE 12 PAGE 1 01/23/97 14:26:51

LOT NUMBER:____________ EXP. DATE:____________ USER:____________

WAVELENGTHS=405NM 630NM

	1	2	3	4	5	6	7	8	9	10	11	12
1A	0.085	1.057	0.230	0.333	0.366	0.099	0.083	0.076	0.042	0.055	0.053	0.078
	Nly	A12Std	linker	3F2	3F2'	3F2²	3F2³	HA6	HA8	HA9	HA12	HB2
1B	0.076	0.120	0.089	0.056	0.122	0.188	0.095	0.056	0.056	0.067	0.061	0.056
	HB3	HB4	HC2	HC4	HD1	HD3	HD7	HE2	HF10	HG4	HG5	HH7
1C	0.056	0.072	0.138	0.068	0.057	0.290	0.055	0.061	0.145	0.055	0.059	0.082
	5A8	5A12	5B3	5B6	5C3	5C7	5C12	5D6	5D12	5F3	5F8	5F12
1D	0.085	0.062	0.095	0.078	0.064	0.068	0.057	0.093	0.074	0.100	0.079	0.245
	5G2	5G6	5G7	5G9	5G12	5H6	5H7	5H9	5H10	6A2	6A3	6A6
1E	0.087	0.071	0.108	0.259	0.084	0.114	0.088	0.111	0.134	0.149	0.103	0.094
	6B11	6C11	6D4	6D9	6D10	6D12	6E2	6F2	6G4	6G6	6H8	6H12
1F	-0.002	-0.002	-0.001	-0.001	-0.002	-0.001	-0.001	-0.001	-0.001	-0.001	-0.001	-0.002

END OF RUN

STAT FAX 2100 :S SN 1112 01/23/97 14:29:49

ABSORBANCE MODE 12 PAGE 1 01/23/97 14:29:58

LOT NUMBER:____________ EXP. DATE:____________ USER:____________

WAVELENGTHS=450NM 630NM

	1	2	3	4	5	6	7	8	9	10	11	12
1A	0.139	2.387	0.487	0.731	0.814	0.174	0.138	0.118	0.043	0.068	0.065	0.120
1B	0.121	0.220	0.154	0.071	0.227	0.385	0.164	0.064	0.067	0.086	0.079	0.075
1C	0.069	0.119	0.277	0.108	0.082	0.643	0.074	0.093	0.293	0.070	0.072	0.128
1D	0.143	0.090	0.170	0.128	0.095	0.088	0.078	0.163	0.114	0.176	0.130	0.530
1E	0.151	0.114	0.197	0.563	0.141	0.213	0.153	0.206	0.268	0.300	0.171	0.169
1F	-0.001	-0.001	-0.000	-0.000	0.000	-0.001	0.000	-0.001	-0.000	-0.000	0.000	-0.000

END OF RUN

STAT FAX 2100 :S SN 1112 01/23/97 14:32:26

3/21/97

	1	2	3	4	5	6	7	8	9	10	11	12
A									Hy	Alz Sta serum	1B7	1C9
B	2B8	2H6	3F2	3F12	4D3	5C7	6A6	6D9	7C7	11E9	4D3^2	7C7^2
C	2B8^2	6A6^2	3F2^2	3F12^2	11E9^2	1B7^2	1C9^2	6D9^2	5C7^2	2H6^2	5C7^3	11E9^3
D												
E	Hy	Alz Sta serum	1B7	1C9	2B8	2H6	3F2	3F12	4D3	5C7		
F	6A6	6D9	7C7	11E9	4D3^2	7C7^2	2B8^2	6A6^2	3F2^2	3F12^2	11E9^2	1B7^2
G	1C9^2	6D9^2	5C7^2	2H6^2	5C7^3	11E9^3						
H												

Alz Sta + 1-40 (rows A–C)

1-43 (rows E–G)

15ul 50ul/well

Testing supernatants to check if still +
Check on Alz Sta, Alz 1-40, + 1-43 plates

Results:

Alz Sta	Alz 1-40	1-43
11E9	3F2	6H6
	6A6	3F2
	7C7^2	7C7, 7C7^2

B001634

ABSORBANCE MODE 12 PAGE 1 03/21/97 15:35:27

LOT NUMBER:________________EXP. DATE:____________________USER:______________________________

WAVELENGTHS=450NM 630NM

	1	2	3	4	5	6	7	8	9	10	11	12	
1A	-0.000	-0.000	-0.000	0.000	-0.000	0.000	-0.000	-0.001	0.096 Hy	1.787 Alz Sta Serum	0.107 1B7	0.113 1C9	
1B	0.105 2B8	0.234 2H6	0.576 3F2	0.409 3F12	0.323 4D3	0.585 5C7	0.847 6A6	0.137 6D9	0.646 7C7	2.245 11E9	0.244 4D3^{2}	1.245 7C7^{2}	Alz Sta
1C	0.157 2B8^{2}	0.333 6A6^{2}	0.306 3F2^{2}	0.274 3F12^{2}	2.217 11E9^{2}	0.104 1B7^{2}	0.082 1C9^{2}	0.108 6D9^{2}	0.497 5C7^{2}	0.236 2H6^{2}	0.230 5C7^{3}	2.244 11E9^{3}	
1D	0.003	0.001	0.002	0.001	0.005	0.006	0.006	0.003	0.004	0.004	0.002	0.001	
1E	0.000	0.000	-0.000	0.000	0.000	0.000	0.000	0.000	0.054 Hy	0.290 Alz Sta Serum	0.045 1B7	0.057 1C9	
1F	0.214 2B8	0.351 2H6	1.205 3F2	0.422 3F12	0.185 4D3	0.626 5C7	1.562 6A6	0.131 6D9	0.928 7C7	0.179 11E9	0.313 4D3^{2}	1.572 7C7^{2}	Alz 1-HC
1G	0.273 2B8^{2}	0.732 6A6^{2}	0.725 3F2^{2}	0.289 3F12^{2}	0.199 11E9^{2}	0.176 1B7^{2}	0.135 1C9^{2}	0.122 6D9^{2}	0.537 5C7^{2}	0.229 2H6^{2}	0.441 5C7^{3}	0.206 11E9^{3}	
1H	0.001	-0.001	-0.000	-0.000	0.000	-0.000	-0.001	-0.002	-0.003	-0.002	-0.002	-0.001	

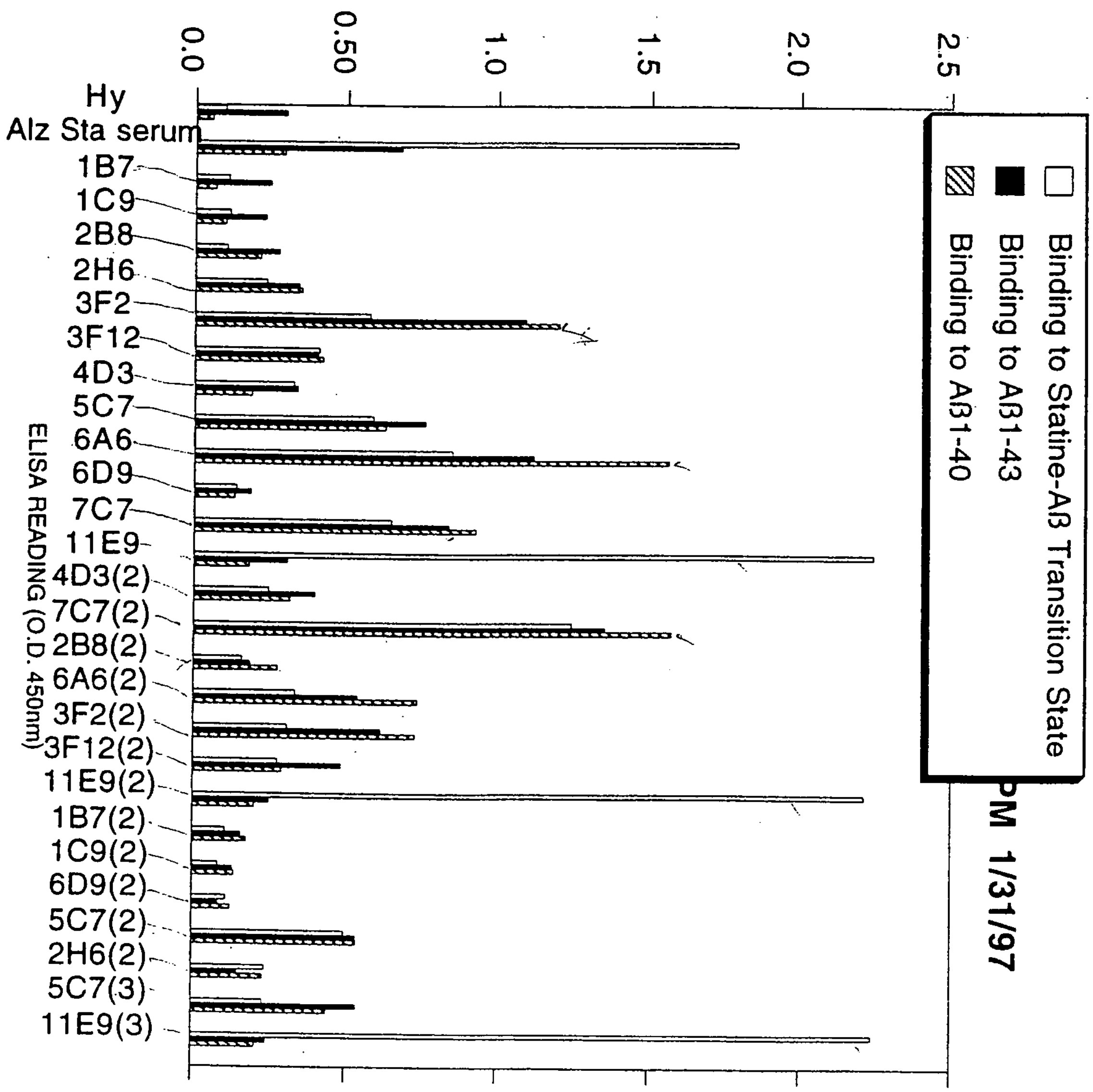
END OF RUN

	1	2	3	4	5	6	7	8	9	10	11	12
2A	0.297 Hy	0.676 Alz Sta Serum	0.246 1B7	0.229 1C9	0.276 2B8	0.340 2H6	1.093 3F2	0.403 3F12	0.335 4D3	0.757 5C7	0.000	0.000
2B	1.118 6A6	0.183 6D9	0.834 7C7	0.306 11E9	0.396 4D3^{2}	1.355 7C7^{2}	0.183 2B8^{2}	0.538 6A6^{2}	0.612 3F2^{2}	0.484 3F12^{2}	0.248 11E9^{2}	0.156 1B7^{2}
2C	0.131 1C9^{2}	0.084 6D9^{2}	0.335 5C7^{2}	0.146 2H6^{2}	0.537 5C7^{3}	0.242 11E9^{3}	-0.001	-0.000	-0.000	-0.001	-0.001	-0.000
2D	0.005	0.006	-0.003	0.008	-0.004	0.007	-0.000	-0.001	-0.001	-0.001	-0.001	-0.000

END OF RUN

B001635

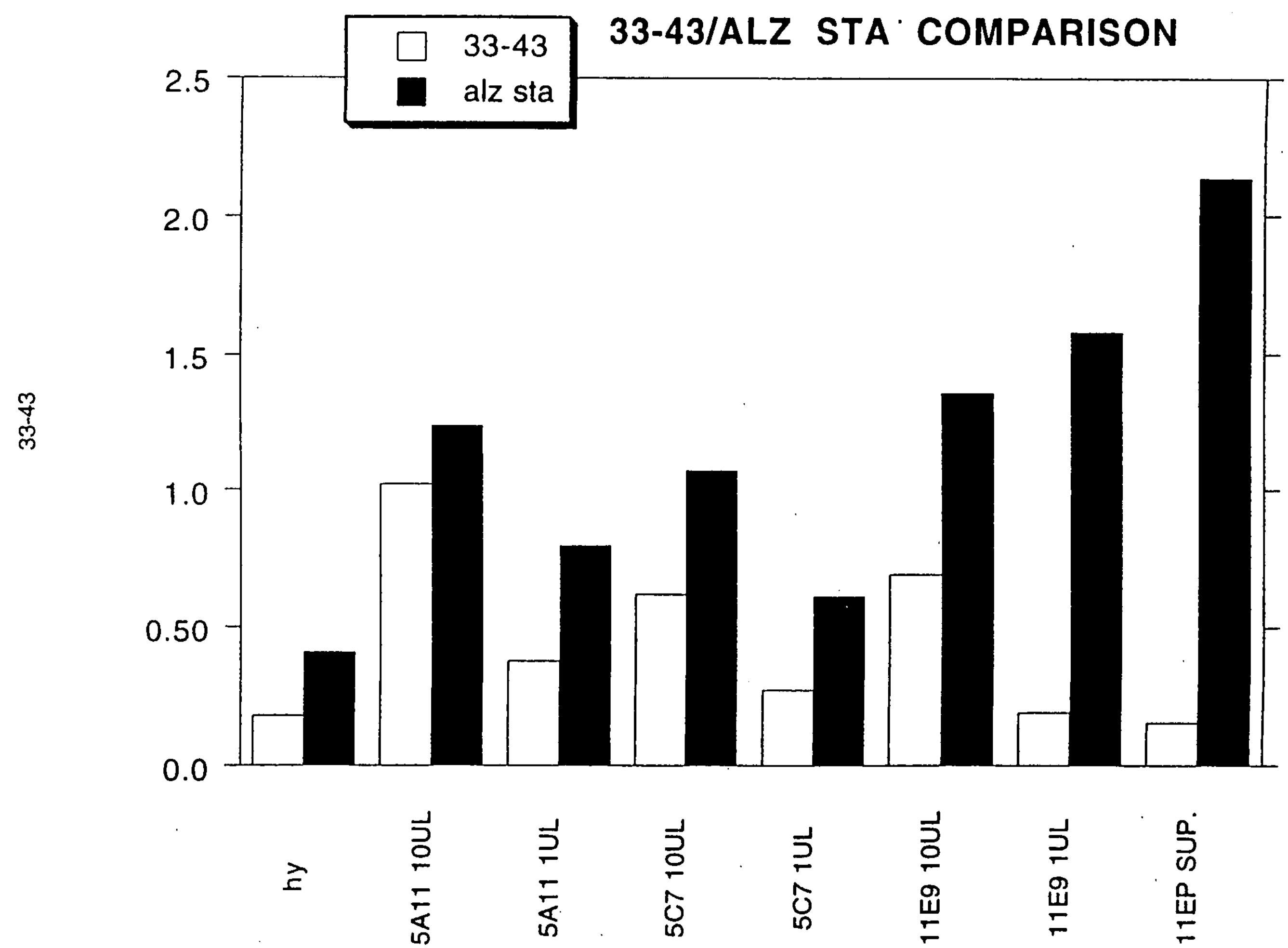
Binding to Statine-Aβ Transition State



4/7/97

	1	2	3	4	5	6	7	8	9	10	11	12	
A	Hy	BS 10uL + 5A11	BS 1uL + 5A11	+ 10uL 5C7	+ 1uL 5C7	+ 10uL 11E9	- 1uL 11E9	- 100uL 11E9 sup					33-013
B													
C	Hy	BS + 5A11	BS + 5A11	+ 5C7	+ 5C7	++ 11E9	++ 11E9	++ 11E9 sup					Alz Std
D													
E													
F													
G													
H													

* testing ascites


33-43/ALZ STA COMPARISON
33-43
alz sta
2.5
2.0
1.5
1.0
0.50
0.0
33-43
hy
5A11 10UL
5A11 1UL
5C7 10UL
5C7 1UL
11E9 10UL
11E9 1UL
11EP SUP.

B001638

STAT FAX 2100 :S SN 1112 04/07/97 14:59:44

04/07/97 14:59:51

ABSORBANCE MODE 12 **PAGE** 1

LOT NUMBER:________ EXP. DATE:________ USER:________

WAVELENGTHS=450NM 630NM

	1	2	3	4	5	6	7	8	9	10	11	12	
1A	0.181	1.020	0.378	0.618	0.275	0.693	0.193	0.157	0.000	-0.001	-0.000	-0.000	33-H3
	H4	5A11	5A11	5C7	5C7	11E9	11E9	11E9 Sup					
1B	-0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	-0.000	0.000	0.000	0.000	
1C	0.410	1.236	0.797	1.071	0.610	1.356	1.581	2.137	0.000	-0.000	-0.000	-0.001	A1z Sk
	H4	5A11	5A11	5C7	5C7	11E9	11E9	11E9 Sup					
1D	-0.001	0.000	-0.000	-0.001	-0.000	-0.000	-0.001	-0.000	-0.000	-0.001	-0.001	-0.000	

END OF RUN

B001639

4/22/97
#12 Sta Clones

plate #1

#12 Sta plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	1A1	1A2	1A3	1A4	1A5	1A6	1A7	1A8	1A9	1A10	1A11	1A12
B	+/- 1B1	1B2	1B3	1B4	+ 1B5	1B6	1B7	1B8	1B9	1B10	1B11	1B12
C	1C1	+/- 1C2	1C3	1C4	1C5	1C6	1C7	1C8	1C9	1C10	1C11	1C12
D	1D1	1D2	1D3	1D4	1D5	1D6	1D7	1D8	1D9	+/- 1D10	1D11	1D12
E	1E1	1E2	1E3	1E4	1E5	1E6	1E7	1E8	1E9	1E10	1E11	1E12
F												
G												
H												

*Multiple clones in wells, separate (+) clones

+

separate
- 1B5 + (hottest clone)
- 1B1 +/-
- 1C2 +/-
- 1D10 +/-

B001640

4/22/97
Pz5A clones

use #1 + 2
1b sta plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	1F1	1F2	1F3	1F4	1F5	1F6	1F7	1F8	1F9	1F10	1F11	1F12
B	1G1	1G2	1G3	1G4	1G5	1G6	1G7	1G8	1G9	1G10	1G11	1G12
C	1H1	1H2	+/- 1H3	1H4	1H5	1H6	1H7	1H8	1H9	1H10	1H11	1H12
D	2A1	2A2	2A3	2A4	2A5	2A6	2A7	2A8	2A9	2A10	2A11	2A12
E	2B1	2B2	2B3	2B4	2B5	2B6	2B7	2B8	2B9	2B10	2B11	2B12
F	2C1	2C2	2C3	2C4	2C5	2C6	2C7	2C8	2C9	2C10	2C11	2C12
G	2D1	2D2	2D3	2D4	2D5	2D6	2D7	2D8	2D9	2D10	2D11	2D12
H	2E1	+ 2E2	2E3	2E4	2E5	2E6	2E7	2E8	+/- 2E9	2E10	2E11	2E12

Multiple clones in wells, separate + clones

+

1H3 (+/-)
2C2 (+) hottest
2E9 (+/-)

B001641

A12 Sta Clones

plate #'s 2+3

A12 Sta plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	2F1	2F2	2F3	2F4	2F5	2F6	2F7	2F8	2F9	2F10	2F11	2F12
B	2G1	2G2	2G3	2G4	2G5	2G6	2G7	2G8	2G9	2G10	2G11	2G12
C	2H1	2H2	2H3	2H4	2H5	2H6	2H7	2H8	2H9	2H10	2H11	2H12
D	+ 3A1	3A2	3A3	3A4	3A5	3A6	3A7	3A8	3A9	3A10	3A11	3A12
E	3B1	3B2	3B3	3B4	3B5	+ 3B6	3B7	3B8	3B9	3B10	3B11	3B12
F	3C1	3C2	3C3	3C4	+ 3C5	3C6	3C7	3C8	3C9	3C10	3C11	3C12
G	3D1	3D2	3D3	3D4	3D5	3D6	3D7	3D8	3D9	3D10	3D11	3D12
H	3E1	3E2	3E3	3E4	3E5	3E6	3E7	3E8	3E9	3E10	3E11	3E12

+

3A1
3B6
3C5

Separate multiple clones

B001642

4/23/97
A12 Sta clones

plate #'s 3 + 4
A12 Sta plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	3F1					3F6						3F12
B	3G1											3G12
C	+ 3H1											3H12
D	4A1					+ 4A6						4A12
E	4B1							+ 4B8				4B12
F	4C1										+ 4C11	4C12
G	4D1											4D12
H	4E1						+ 4E7					4E12

+

3F6
3H1
4A6
4B8
4C11
4E7

K12 Sta Clones 4/24/97

	1	2	3	4	5	6	7	8	9	10	11	12
A	1B1	1B5	1C2	1D10	1H3	2E2	2E9	3A1	3B6	3C5	3F6	3H1
B												
C	1B1	1B5	1C2	1D10	1H3	2E2	2E9	3A1	3B6	3C5	3F6	3H1
D												
E												
F												
G												
H												

K12 Sta plate ⊕ clones

33-43 plate

K12 Sta Results

1B7
3B6
3C5 } hottest

(not obviously)

33-43 peptide Results

1B1
1B5
1C2 } slightly weak

4/24/97
A12 Sta Clones

plate #'s 4-5
A12 Sta plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	+ 4F1								+ 4F9			4F12
B	4G1											4G12
C	4H1											4H12
D	5A1											5A12
E	5B1						+ 5B7					5B12
F	5C1				+ 5C5							5C12
G	5D1											5D12
H	5E1				+ 5E5							5E12

+

4F1
4F9
5B7
5C5
5E5

11/24/97
A12-5ta [illegible]

		1	2	3	4	5	6	7	8	9	10	11	12
A12-5ta plasma	A	HV	4F1	4F9	5E7	5E5	5E5						
	B		+++	++	++++	+++	+++						
1-413	C	HY	4F1	4F9									
	D	5E5	5E5	5E5									
	E		++										
	F												
	G												
	H												

B001646

4/22/97
Pos Ab [illegible]

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C	5F1											5F12
D	5G1											5G12
E	5H1											5H12
F	6A1						+ 4A7					6A12
G	6B1											6B12
H	6C1		+ 6C3									6C12

1025574le
A12016 plate

Results

4A7
6C3

11/21/07

Hz Sta Clones

plate #'s 6+7

Hz Sta plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	6D1			+ 6D4								6D12
B	6E1									+ 6E10	+ 6E11	6E12
C	6F1										+ 6F11	6F12
D	6G1											6G12
E	6H1											6H12
F	7A1											+ 7A12
G	7B1									+ 7B10		7B12
H	7C1											7C12

Results

6D4 ++
6E10 +++ (hottest)
6E11 ++
6F11 ++
7A12 ++
7B10 ++

B001648

4/28/97
Ptz Sto Clones

	1	2	3	4	5	6	7	8	9	10	11	12
A	H4	4A7	6C3	6D4	6E10	6E11	6F11	7H12	7B10			
B												
C												
D												
E												
F												
G												
H												

1-H3 plate

Results:
* No positive Clones

B001649

4P01#?
Ak Sta Clones

Ak Sta plate
plates 7 + 8

	1	2	3	4	5	6	7	8	9	10	11	12
A	7D1											7D12
B	7E1	+ 7E2										7E12
C	7F1	+ 7F2										7F12
D	7G1	+ 7G2			+ 7G5							7G12
E	7H1											7H12
F	8A1			+ 8A4								8A12
G	8B1											8B12
H	8C1											8C12

Results:

7E2
7F2
7G2
7G5
8A4

Ab Sta Clones
1/28/97

1x Sta plate #'s 8 & 9

	1	2	3	4	5	6	7	8	9	10	11	12
A	8D1											8D12
B	8E1											8E12
C	+ 8F1	+ 8F2										8F12
D	8G1											8G12
E	8H1								+ 8H9			8H12
F	9A1											9A12
G	9B1											9B12
H	9C1											9C12

Results:

8F1
8F2
8H9

4/9/97
A12 Sta Clones

	1	2	3	4	5	6	7	8	9	10	11	12
A				H4	7E2	7F2						
B	7G2	7G5	(+/-) 8H4	(+) 8F1	8F2	8H9						
C												
D												
E												
F												
G												
H												

1-H3 plate

Results:

8F1
8H4 +/-

Hz Std Clones
4/29/97

Hz Std plate #'s 9 & 10

	1	2	3	4	5	6	7	8	9	10	11	12
A	9D1					+ 9D6	+ 9D7					9D12
B	9E1											9E12
C	9F1											9F12
D	+ 9G1						+ 9G7					9G12
E	9H1											9H12
F	10A1						+ 10A7					10A12
G	10B1											10B12
H	10C1									+ 10C10		10C12

Results:

9D6
9D7
9G1 (hottest)
9G7
10A7
10C10

B001653

4/24/97
A12 Sta Clones

A12 Sta plate #'s 10 & 11

	1	2	3	4	5	6	7	8	9	10	11	12
A	10D1											+ 10D12
B	10E1											10E12
C	10F1											10F12
D	10G1								+ 10G9			10G12
E	10H1			+/- 10H4								10H12
F	+ 11A1											+ 11A12
G	11B1			+/- 11B4		+/- 11B6						11B12
H	11C1											11C12

A12 Sta plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	11D1				+ 11D5							11D12
B	11E1		+ 11E3									11E12

Results:

+	
10D12	10H4
10G9	11B4
11A1	11B6
11A12	11D5
	11E3

+/- *only* seen 1-A3 plate

B001654

[illegible]

Ab 5 to Clones

	1	2	3	4	5	6	7	8	9	10	11	12
A	Hy	9D6	9D7	9G1	9G7	10F7						
B	10C10	10D12	10G9	10H4	11A1	11A6	11B4	11B6	11D5	11E3		
C												
D												
E												
F												
G												
H												

1-H3 plate

Results:

* No positive clones

5/5/97
A12Sta Clones

	1	2	3	4	5	6	7	8	9	10	11	12
A	Hy	1C2 1	1C2 2	1C2 3	1C2 4	1C2 5	1D10 1	1D10 2	1D10 3	1D10 4	1H3 1	1H3 2
B	3F6 1	3F6 2	3F6 3	3F6 4	4B8 1	4B8 2	4B8 3	4B8 4	4E7 1	4E7 2	4E7 3	4E7 4
C	4F9 1	4F9 2	4F9 3									
D												
E												
F												
G												
H												

A12Sta plate

Re-Testing clones that were separated (these clones did not give results when 1st tested after the separation)

Results:

4E7[1] +/-
4E7[2] +/- ?

No other positive clones showed up.

5/5/97
A12Sta Clones

1-H3 plate

	1	2	3	4	5	6	7	8	9	10	11	12
A											HY	1D1[2]
B				1B5[3]	2E2[4]	2E9[5]	3A1[3]	3B6[1]	3C5[1]	3H1[1]	4A6[3]	4C11[1]
C												
D												
E												
F												
G												
H												

* took 10uL from ⊕ clones that were separated

Results:

4A6 (very light yellow)
only one to show up ⊕

4/30/91
Ab Stc Clones

1b Sta plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Hy	1B1 1	+ 1B1 2	1B1 3	1B5 1	1B5 2	+ 1B5 3	1B5 4	1C2 1	1C2 2	1C2 3	1C2 4
B	1C2 5	1D10 1	1D10 2	1D10 3	1D10 4	1H3 1	1H3 2	2E2 1	2E2 2	2E2 3	+L 2E2 4	2E2 5
C	2E2 6	2E9 1	2E9 2	2E9 3	2E9 4	+L 2E9 5	3A1 1	3A1 2	+ 3A1 3	+ 3B6 1	3B6 2	3B6 3
D	3B6 4	+ 3C5 1	3C5 2	3C5 3	3C5 4	3C5 5	+ 3H1 1	3H1 2	3H1 3	3H1 4	3F6 1	3F6 2
E	3F6 3	3F6 4	4A6 1	4A6 2	+ 4A6 3	4B8 1	4B8 2	4B8 3	4B8 4	+ 4C11 1	4C11 2	4C11 3
F	4E7 1	4E7 2	4E7 3	4E7 4	+ 4F1 1	4F1 2	4F1 3	4F1 4	4F9 1	4F9 2	4F9 3	
G												
H												

* testing (+) clones that were separated

Results:

1B1 2
1B5 3
3A1 3
3B6 1
3C5 1
3H1 1
4A6 3
4C11 1
4F1 1

2E2 4 (+L)
2E9 5 (+L)

Retest

1C2
1D10
1H3

3F6
4B8
4E7
4F9

B001658

Alz Sta Clones
5/6/97

Alz Sta plate

	1	2	3	4	5	6	7	8	9	10	11	12
A				Hy	$5B7^{1}$	$5B7^{2}$	$5B7^{3}$	$5C5^{1}$	$5C5^{2}$	$5C5^{3}$	$5C5^{4}$	$5E5^{1}$
B	$5E5^{2}$	$5E5^{3}$	+++ $6A7^{1}$	$6A7^{2}$	$6A7^{3}$	$6A7^{4}$	$6C3^{1}$	$6C3^{2}$	$6C3^{3}$	$6C3^{4}$	$6C3^{5}$	$6D4^{1}$
C	$6D4^{2}$	$6E10^{1}$	$6E10^{2}$	+++ $6E10^{3}$	$6E11^{1}$	$6E11^{2}$	$6E11^{3}$	$6F11^{1}$	$6F11^{2}$	$7A12^{1}$	$7A12^{2}$	$7A12^{3}$
D	$7B10^{1}$	+++ $7B10^{2}$	+++ $7B10^{3}$	$7B10^{4}$	$7E2^{1}$	+++ $7E2^{2}$	+++ $7F2^{1}$	$7F2^{2}$				
E												
F												
G												
H												

Results

+	−
$6A7^{1}$ +++	5B7
$6E10^{3}$ +++	5C5
$7B10^{2}$ +++	5E5
$7E2^{2}$ +++	6D4
$7F2^{1}$ +++	6F11
$7A12^{2}$ +	
$6E11^{2}$ +	
$6C3^{4}$ +	

B001659

5/8/97

A12 5ta Clones

A12 5ta plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Hy	$7G2^{1}$	$7G2^{2}$	$7G5^{1}$	$7G5^{2}$	$7G5^{3}$	$7G5^{4}$	$8A4^{1}$	$8A4^{2}$	$8F1^{1}$	$8F1^{2}$	$8F1^{3}$
B	$8F2^{1}$	$8F2^{2}$	(+) $8H9^{1}$	$8H9^{2}$	$9D6^{1}$	$9D6^{2}$	(+) $9D6^{3}$	$9D6^{4}$	$9D7^{1}$	$9D7^{2}$	$9D7^{3}$	$9G1^{1}$
C	$9G1^{2}$	$9G7^{1}$	$9G7^{2}$	$9G7^{3}$	$10A7^{1}$	$10A7^{2}$	$10A7^{3}$	$10A7^{4}$	$10A7^{5}$	$10C10^{1}$	$10C10^{2}$	$10C10^{3}$
D	$10D12^{1}$	$10D12^{2}$	$10D12^{3}$	$10G9^{1}$	$10G9^{2}$	(+) $11A1^{1}$	$11A1^{2}$	$11A12^{1}$	$11A12^{2}$			
E												
F												
G												
H												

Results:

$8H9^{1}$ +

$9D6^{3}$ +/-

$11A1^{1}$ ++

5/9/97
A12 Sta Clones

A12 Sta plate

	1	2	3	4	5	6	7	8	9	10	11	12
A										H4	1C2	1C2
B	1C2	1C2	1C2	1D10	1D10	1D10	1D10	4H3	4H3	3F6	3F6	3F6
C	3F6	4B8	4B8	4B8	4B8	4E7	4E7	4E7	4E7	4F9	4F9	4F9
D	5B7	+ 5B7	5B7	5C5	5C5	5C5	+ 5C5	5E5	5E5	+ 5E5	+ 6D4	6D4
E	+ 6F11	6F11										
F												
G												
H												

Retesting A12 Sta clones that were separated when they were first tested the clones came up negative.

Results:

5B7^{2}
5C5^{4}
5E5^{3}
6D4^{1}
6F11^{1}

5/15/97
A12 Sta

A12 Sta plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	7G2	7G2	7G5	7G5	7G5	7G5	8AH	+ 8AH	+ 8F1	8F1	8F1	+ 8F2
B	8F2	9D7	9D7	9D7	9G1	+ 9G1	9G7	9G7	9G7	10A7	+ 10A7	10A7
C	10A7	10A7	10C10	10C10	10C10	10D12	10D12	10D12	10G9	+ 10G9	11A12	11A12
D												
E												
F												
G												
H												

*testing separated clones

10G 9^2 ++
10A 7^2 ++
8AH^2 +/-
8F 1' +/-
8F2' +/-

5/23/97
H2 5ta

	1	2	3	4	5	6	7	8	9	10	11	12
A	6A7	6C3	+/- 6E10	6E11	7A12	+/- 7B10	7F2	8H9	+ 9D6	+ 3B6	± 2E2	± 2E9
B	+ 8A4	H4										
C												
D												
E												
F												
G												
H												

H2 5ta plate

Screening H2 5ta Clones in limbro wells

Results

6E10 +/-
7B10 +/-
9D6 +
3B6 +
2E2 +
2E9 +
8A4 +

ANIMAL FACILITY - Internal Requisition Form

No. 5087 **A**

Date: 2/3/97 Protocol #: CMB 0697 Fund #: BBRI

Ship to:	Invoice in duplicate to:
☐ The Schepens ☑ BBRI ☐ Other ______ Attn of: MA ORTEGA	The Schepens Eye Research Institute / Boston Biomedical Research Institute Animal Facility 20 Staniford Street Boston, MA 02114 Phone (617) 742-3140 x 215 Fax (617) 723-1741

REQUISITION TO:

McLaughlin Res. Inst.
1520 23rd St. South
Great Falls, MONTANA 59405

CUBICLE A

(QUARANTINE AREA HOUSING UPON ARRIVAL)

FOR OFFICE USE ONLY
Ordered by: V. Rao/MA Ortega
Vendor Ref #: Sylvia Banks
Delivery Date: Will call. 2/18

Principal Investigator: Vic Rao Ext. — E-Mail —

Date experiment will start: ASAP Contact person: MARIE Ortega Ext. 441

Authorized signature: Vic Rao

Species of animal requested: MICE Strain: Tg (HuAPP695 SWE) 2576

Quantity: 2 Weight: — Sex: ♀ Age: Adult

Special instructions: TOTAL $115.00

Arrived early on 2/18/97 / one day early

INVESTIGATOR

B001734